

**THE ROLE OF THE PRO-APOPTOTIC
BCL-2 FAMILY PROTEINS
IN FOCAL CEREBRAL ISCHAEMIA**

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ABSTRACT

The pathophysiology of stroke is a complex process and the role of apoptosis in ischaemic cell death has been much debated. Bcl-2 family proteins, which regulate mitochondrial function and ultimately cell fate, have been implicated in ischaemic cell death. Although the precise mechanisms of cell death remain unresolved, targeting delayed cell death mechanisms, specifically the Bcl-2 family, may be the therapeutic frontier. The aims of this thesis were to compare and contrast *in vitro* and *in vivo* mechanisms of pro-apoptotic Bcl-2 family protein activation, and to investigate the Bcl-2 family as putative targets in stroke research.

Several *in vitro* models of apoptosis were initially set-up to characterize the mechanism of action of Bax, Bad and Nix, as examples of 'multidomain', 'BH3 domain-only' and 'BH3 / transmembrane domain-containing' pro-apoptotic Bcl-2 family proteins, respectively. Subsequently, several models of focal ischaemia, with varied occlusion and reperfusion durations, were set-up to characterize the putative pro-apoptotic Bcl-2 family response(s) to focal cerebral ischaemia and the effects of reperfusion on ischaemic damage. Subcellular fractionation and confocal microscopy techniques were established *in vitro* and *in vivo* and confirmed to be an accurate method of assessing Bcl-2 protein activity. Finally, the *in vitro* and *in vivo* activation pathways of Bax, Bad and Nix were compared to justify the use of such simplified *in vitro* models of apoptosis for the characterization of the Bcl-2 protein family molecular circuitry.

Staurosporine (5 h/500nM) was used to induce apoptosis in human SH-SY5Y neuroblastoma cells and Bcl-2 family protein activation was investigated. Apoptosis was associated with upregulation of Bax and Nix, as well as upregulation of a novel 30 kDa Nix-like protein. In contrast, apoptosis resulted in dephosphorylation, but not upregulation, of Bad. Mitochondrial accumulation appeared to be a common feature of these proteins' pro-apoptotic response. This was the first study to demonstrate that the hypoxia-responsive Nix could be activated by stimuli other than chronic hypoxia. Interestingly, upregulation, but not translocation of Nix was observed *in vitro* for each treatment group. Apoptosis-mediated attachment to and insertion into the outer mitochondrial membrane was observed for Bad and Bax, respectively. This supports the theory that membrane insertion is dependent on the presence of a transmembrane domain.

Similarly Bax, Bad and Nix activation pathways were investigated *in vivo* following focal cerebral ischaemia. However, different pro-apoptotic protein responses were observed after permanent and transient middle cerebral artery (MCA) occlusion. Mitochondrial accumulation of Bax and Nix was

observed with increased ischaemic duration in the permanent model. Bad dephosphorylation, but not translocation, was observed with increased ischaemic duration. An antibody problem was likely the explanation for the lack of observed Bad translocation, since the whole cell lysate fraction of Bad levels remained relatively constant, in contrast to the reduced cytosolic levels of Bad observed with increased ischaemic duration. Interestingly, these events peaked 24 h after ischaemic onset, particularly in the striatum, an area considered to undergo rapid necrotic-like cell death. In contrast, a novel Akt-regulated Bad inactivation was detected with increased reperfusion duration.

The present study identified different pro-apoptotic Bcl-2 family responses to permanent and transient MCA occlusion. In contrast to the 'reperfusion damage' theory, the present study reported an Akt-mediated Bad inactivation which might reflect a novel intrinsic survival response during extended periods of reperfusion. Targeting the Akt-Bad pathway might provide a novel therapeutic target for stroke research. In conclusion, data presented in this thesis therefore support a role for the Bcl-2 family proteins in focal cerebral ischaemia.

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TO THE MEMORY
OF MY GRANDFATHER

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DECLARATION

The candidate confirms that the work submitted is her own and that appropriate credit has been given where reference has been made to the work of others.

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PUBLICATIONS ARISING FROM THESIS

PAPERS

Birse-Archbold J-LA, Kerr LE, Sharkey J and J McCulloch (2003). Multidomain and BH3 domain-only Bcl-2 family responses to staurosporine treatment in human neuroblastoma cells. (*In preparation*).

Birse-Archbold J-LA, Kerr LE, PA Jones, Sharkey J and J McCulloch (2003). Changes in the subcellular distribution of Akt and pro-apoptotic Bcl-2 family protein, Bad, reflect a novel survival mechanism following transient focal cerebral ischaemia in the rat. (*In preparation*).

Birse-Archbold J-LA, Kerr LE, PA Jones, Sharkey J and J McCulloch (2003). Upregulation and translocation of pro-apoptotic Bcl-2 family proteins Bax and Nix following permanent focal cerebral ischaemia in the rat. (*In preparation*).

Birse-Archbold J-LA, Kerr LE, PA Jones, Sharkey J and J McCulloch (2003). *In vitro* models of apoptosis and *in vivo* models of focal cerebral ischaemia support a model where Akt detachment, Bad attachment and Bax insertion into the outer mitochondrial membrane coincides with caspase-3 cleavage and morphological features of apoptotic cell death. (*In preparation*).

ABSTRACTS & POSTERS

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LIST OF FIGURES

Figure 1.2.1: Simplified diagram showing the cerebral circulation of the rat.	3
Figure 1.2.2: Viability thresholds of cerebral ischaemia.	4
Figure 1.2.3: Monofilament occlusion model of middle cerebral artery occlusion.	7
Figure 1.2.4: The Ischaemic Cascade.	9
Figure 1.3.1: Schematic diagram of oxidative phosphorylation and ATP generation in the mitochondria.	12
Figure 1.3.2: Mitochondrial-mediated cell death cascade; formation of the apoptosome.	14
Figure 1.4.1: Classification of the Bcl-2 protein family.	18
Figure 1.4.2: Bcl-2 family protein regulation – upstream of irreversible cell death.	21
Figure 1.5.1: Bcl-2 family regulation of ischaemic cell death.	25
Figure 2.1: Relationship between ischaemic duration and severity of insult.	28
Figure 2.2: Placement of intraluminal monofilament for permanent and transient MCA occlusion	31
Figure 2.3: Surgical exposure of the arteries in the neck of the rat.	32
Figure 2.4: Placement of the monofilament in the vasculature of the mouse brain such that it occludes the origin of the middle cerebral artery.	32
Figure 2.5: <i>Distribution of damage in a model of transient ischaemic stroke</i>	34
Figure 2.6: Typical distribution of damage following MCA occlusion.	34
Figure 2.7: Morphological assessment of cortex 24h after onset of transient (2 h) MCA occlusion	35
Figure 2.8: Volume of Damage observed 24 h and 72 h after onset of Permanent MCA occlusion	36
Figure 2.9: Volume of Damage observed 24 h and 72 h after onset of Transient (2 h) MCA occlusion	36
Figure 2.10: Protein expression levels of Bax 24 h after transient (2 h) MCA occlusion	37

Figure 2.11: Analysis of Bad protein expression 24 h after transient (2 h) MCA occlusion	38
Figure 2.12: Analysis of Nix protein expression 24 h after transient (2 h) MCA occlusion	38
Figure 2.13: Cleaved caspase-3 protein expression 24 h after transient (2 h) MCA occlusion	39
Figure 3.1: Confirmation of apoptosis in human SHSY-5Y, mouse A1.1 and rat CGC cells	51
Figure 3.2: Upregulation of Bax in STS-, DEX- and KSD-treated cells	51
Figure 3.3: Translocation of Bax in STS- and DEX-treated cells	52
Figure 3.4: STS-induced apoptosis of SH-SY5Y cells involves apoptosome formation	53
Figure 3.5: Time course of cell death in STS-treated SH-SY5Y cells	54
Figure 3.6: STS-mediated changes in of SH-SY5Y cell morphology	54
Figure 3.7: Visualisation of STS-induced mitochondrial changes using MitoTracker CMXRos	55
Figure 3.8: Analysis of STS-mediated changes in mitochondrial inner membrane (IMM) potential using potential-sensitive dye, JC-1	56
Figure 3.9: STS-mediated redistribution of Bax and cytochrome c.	57
Figure 3.10: Quantitative analysis of STS-mediated cytochrome c release	58
Figure 3.11: STS-mediated changes in Bax association at the OMM	60
Figure 3.12: Cyclosporine A-mediated dose-dependent inhibition of cytochrome c release	61
Figure 3.13: The mitochondrial permeability transition pore	65
Figure 4.1: STS-mediated changes in phosphoserine136, but not total Bad	73
Figure 4.2: Apoptosis-induced dephosphorylation and mitochondrial accumulation of total Bad	73
Figure 4.3: Apoptosis promotes Bad attachment, but not insertion, at the OMM of SH-SY5Y cells	74
Figure 4.4: STS-mediated changes in total and phosphoserine (P) ⁴⁷³ Akt	75

Figure 4.5:	Loss of cytosolic and mitochondrial p ⁴⁷³ Akt immunoreactivity in apoptotic SH-SY5Y cells	75
Figure 4.6:	Apoptosis-induced detachment of P ⁴⁷³ Akt from the OMM of SH-SY5Y cells	76
Figure 5.1:	Confirmation of apoptosis in hypoxic CHO-K1 cells	85
Figure 5.2:	Hypoxia-mediated changes in Glut-1 protein expression in CHO-K1 cells	85
Figure 5.3:	Hypoxia-induced changes in Nix protein expression	86
Figure 5.4:	Hypoxia-induced changes in Nix subcellular localisation	87
Figure 5.5:	STS- and DEX-mediated changes in Nix protein expression levels	88
Figure 5.6:	STS-mediated changes in the subcellular distribution of pro-apoptotic Nix	88
Figure 5.7:	BNip3 and Nix sequence alignment.	90
Figure 6.1:	Thionin stained brain sections after permanent MCA occlusion in rats.	98
Figure 6.2:	Volume of tissue damage observed with increased ischaemic duration.	99
Figure 6.3:	H&E staining of the ischaemic lesion after 24 h permanent ischaemia.	100
Figure 6.4:	Peri-vascular response in the cortex and striatum after 6 h and 24 h permanent ischaemia.	100
Figure 6.5:	Peri-vascular response in the cortex after 24 h permanent ischaemia.	101
Figure 6.6:	Changes in Bax and Nix protein expression after 6 h and 24 h permanent ischaemia.	102
Figure 6.7:	Changes in the subcellular distribution of Bax and Nix with increased ischaemic duration	103
Figure 6.8:	Bad protein expression and phosphorylation status with increased ischaemic duration	104
Figure 6.9:	Assessment of Bad and P ¹³⁶ Bad subcellular distribution after 6 h and 24 h permanent ischaemia.	105
Figure 6.10:	Akt and P ⁴⁷³ Akt protein expression after 6 h and 24 h permanent ischaemia in rats.	105
Figure 6.11:	Assessment of P ⁴⁷³ Akt subcellular distribution after 6 h and 24 h permanent ischaemia.	106

Figure 6.12: Cleaved caspase-3 expression and subcellular distribution after permanent ischaemia.	107
Figure 7.1: Thionin stained brain sections following transient MCA occlusion in rats.	117
Figure 7.2: H&E staining of the ischaemic lesion after 6 h transient ischaemia.	118
Figure 7.3: Volume of tissue damage observed with increased reperfusion duration.	119
Figure 7.4: Comparison of volume of tissue damage observed after permanent and transient ischaemia.	120
Figure 7.5: H&E staining of the ischaemic lesion after 24 h transient ischaemia.	121
Figure 7.6: Peri-vascular response in the striatum and deeper cortex after 24 h transient ischaemia.	121
Figure 7.7: Changes in Bax protein expression after 6 h and 24 h transient ischaemia.	122
Figure 7.8: Changes in the subcellular distribution of Bax following transient ischaemia.	123
Figure 7.9: Changes in Nix protein expression after 6 h and 24 h transient ischaemia.	123
Figure 7.10: Changes in the subcellular distribution of Nix following transient ischaemia.	124
Figure 7.11: Bad protein expression and phosphorylation status after transient ischaemia.	125
Figure 7.12: Subcellular distribution of Bad and P ¹³⁶ Bad 6 h and 24 h after transient ischaemia.	125
Figure 7.13: Akt and P ⁴⁷³ Akt protein expression following transient focal cerebral ischaemia	126
Figure 7.14: Assessment of P ⁴⁷³ Akt subcellular distribution after 6 h and 24 h transient ischaemia.	127
Figure 7.15: Cleaved caspase-3 expression and subcellular distribution after transient ischaemia.	128
Figure 8.1: Schematic representation of thesis.	137
Figure 8.2: Schematic diagram representing observed pro-apoptotic Bcl-2 family response to focal cerebral ischaemia in the rat.	150
Figure 8.3: Schematic diagram of the pathophysiology of focal cerebral ischaemia	158

LIST OF TABLES

Table 1.2.1: Models of middle cerebral artery occlusion in the rat.	8
Table 1.2.2: Pathophysiology of focal cerebral ischaemia	10

TABLE OF CONTENTS

ABSTRACT	II
ACKNOWLEDGMENTS	V
DECLARATION	VI
PUBLICATIONS ARISING FROM THESIS	VII
PAPERS	VII
ABSTRACTS & POSTERS	VII
LIST OF FIGURES	IX
LIST OF TABLES	XIII
ABBREVIATIONS	XIX
CHAPTER 1: GENERAL INTRODUCTION	1
1.1 GENERAL INTRODUCTION	1
1.2 STROKE	2
INCIDENCE & EPIDEMIOLOGY	2
CEREBRAL STRUCTURE	2
CEREBRAL BLOOD FLOW FUNCTION	3
CONCEPTS OF CORE, PENUMBRA & REPERFUSION INJURY	4
REPERFUSION – SAINT OR SINNER?	5
MODELS OF CEREBRAL ISCHAEMIA	5
RODENTS – SUITABLE SPECIES TO MODEL ISCHAEMIA	6
MIDDLE CEREBRAL ARTERY OCCLUSION	6
INTRALUMINAL FILAMENT MCA OCCLUSION	7
PATHOLOGICAL CONSEQUENCES OF FOCAL ISCHAEMIA	9
1.3 MITOCHONDRIAL CONTROL OF CELL FATE	11
ATP PRODUCTION	12
GENERATION OF FREE RADICALS & CALCIUM BUFFERING	13
RELEASE OF APOPTOTIC MEDIATORS	13
APOPTOSOME FORMATION	13

MECHANISMS OF CYTOCHROME C RELEASE	14
MITOCHONDRIAL DYSFUNCTION IN STROKE.....	15
1.4 APOPTOTIC CELL DEATH	16
APOPTOSIS OR NECROSIS: DEFINITIONS & CHARACTERISATION	16
APOPTOSIS – EVOLUTIONARY CONSERVED CELL DEATH MECHANISM.....	17
THE BCL-2 FAMILY	18
THE PROTEINS: MEMBERS & CLASSIFICATION	18
DIMERISATION: CONTROL OVER THE LIFE / DEATH RHEOSTAT.....	19
LEVELS OF REGULATION.....	20
BCL-2 CONTROL OVER THE MITOCHONDRIA	22
1.5 ISCHAEMIC DAMAGE - NECROSIS OR APOPTOSIS?.....	23
CHAPTER 2: FOCAL ISCHAEMIC STROKE	28
INTRODUCTION	28
AIMS	30
MATERIALS & METHODS	30
Rat Permanent Monofilament Occlusion.....	30
Rat Transient Monofilament Occlusion.....	31
Histological Assessment of Damage	32
Biochemical Assessment of Damage.....	33
RESULTS	34
VOLUME & DISTRIBUTION OF DAMAGE.....	34
PRO-APOPTOTIC BCL-2 FAMILY RESPONSE TO FOCAL CEREBRAL ISCHAEMIA ...	37
DISCUSSION	39
CHAPTER 3: <i>IN VITRO</i> CHARACTERISATION OF BAX.....	44
INTRODUCTION	44
AIMS	46
MATERIALS & METHODS	46
CELL CULTURE, DRUG TREATMENT & ANALYSIS OF APOPTOSIS.....	46
Staurosporine-treated human SH-SY5Y neuroblastoma cells	46

Dexamethasone - treated mouse A1.1 monocytes	46
Potassium Serum Deprivation in rat primary cerebellar granule cells	47
ISOLATION OF WHOLE CELL, CYTOSOLIC AND MITOCHONDRIAL FRACTIONS	47
MTS Cell Viability Colorimetric Assay	48
Analysis of Protein Expression by Subcellular Fractionation and Western Blotting.....	48
CONFOCAL MICROSCOPY	48
Visualisation of Bax and Cytochrome C redistribution using Confocal Microscopy	48
ELISA Assay - Quantitative Assessment of Cytochrome C Release	49
Alkali Extraction of Outer Mitochondrial Membrane Proteins	49
Mitotracker Red CMXRos staining of mitochondria in Apoptotic cells	50
Analysis of the Inner Mitochondrial Membrane Potential using JC-1	50
RESULTS	50
DISCUSSION	62
CHAPTER 4: <i>IN VITRO</i> CHARACTERISATION OF BAD.....	69
INTRODUCTION	69
AIMS	71
MATERIALS & METHODS	71
Analysis of Protein Expression by Subcellular Fractionation and Western blotting.....	71
Sodium Carbonate Extraction of Outer Mitochondrial Membrane (OMM) Proteins	72
RESULTS	72
DISCUSSION	77
CHAPTER 5: <i>IN VITRO</i> CHARACTERISATION OF NIX.....	81
INTRODUCTION	81
AIMS	83
MATERIALS & METHODS	83
Induction of apoptosis in Chinese Hamster Ovary cells	83
Analysis of Protein Expression by Subcellular Fractionation and Western blotting.....	84
RESULTS	84
DISCUSSION	89

CHAPTER 6: PERMANENT FOCAL ISCHAEMIA	95
INTRODUCTION	95
AIMS	96
MATERIALS & METHODS	96
Histological Assessment of Damage.....	96
Biochemical Assessment of Damage	96
PREPARATION OF CELL LYSATES.....	96
ISOLATION OF CYTOSOLIC AND MITOCHONDRIAL FRACTIONS	97
RESULTS	98
VOLUME OF DAMAGE.....	98
BCL-2 FAMILY RESPONSE TO PERMANENT ISCHAEMIA	101
DISCUSSION	107
CHAPTER 7: TRANSIENT FOCAL ISCHAEMIA	115
INTRODUCTION	115
AIMS	116
MATERIALS & METHODS	116
RESULTS	117
Histological Assessment of Damage.....	117
Biochemical Assessment of Damage	122
DISCUSSION	129
CHAPTER 8: FINAL DISCUSSION.....	134
EXPERIMENTAL RATIONALE	134
MAJOR FINDINGS.....	138
RAT STROKE MODELS.....	138
IN VITRO REGULATION OF BAX, BAD, & NIX.....	139
STAUROSPORINE-MEDIATED MITOCHONDRIAL DYSFUNCTION & CYTOCHROME C RELEASE	143
<i>IN VIVO</i> MODELS OF FOCAL CEREBRAL ISCHAEMIA	144

INTRINSIC AKT / BAD SURVIVAL RESPONSE – EARLY DURING ISCHAEMIA & WITH PROLONGED REPERFUSION	147
OPEN QUESTIONS AND OUTLOOK	152
REGULATION OF MULTI-DOMAIN AND BH3-DOMAIN ONLY PROTEINS?	152
WHICH CELL TYPE EXPRESSED BAX, BAD, NIX & CLEAVED CASPASE-3?	154
TIME TO RE-DEFINE THE TERMS 'APOPTOSIS' & 'NECROSIS' ?	156
CONCLUSIONS.....	157
APPENDICES.....	159
APPENDIX A: HISTOLOGY	159
CALCULATION OF DAMAGE.....	159
APPENDIX B: COMPUTERISED DIGITAL MORPHOMETRY	160
RAT BRAIN REGIONS	161
STEREOTAXIC TEMPLATES.....	162
SAMPLE EXCEL WORKSHEET	164
APPENDIX C: RUNNING GELS & WESTERN BLOTTING	165
RUNNING SDS-PAGE GELS	165
BUFFERS FOR RUNNING SDS GELS & WESTERN BLOTTING	166
SDS ACRYLAMIDE GELS	167
APPENDIX D: MTS CELL VIABILITY ASSAY	168
BIBLIOGRAPHY.....	169

ABBREVIATIONS

ACA	anterior communicating artery
ADP	adenosine diphosphate
AIF	apoptosis inducing factor
ANT	adenine nucleotide translocator
APAF-1	apoptosis-activating factor-1
ara-c	cytosine β -D-arabino-furanoside
ATP	adenosine triphosphate
ANOVA	analysis of variation
APAF-1	apoptosis protease-activating factor 1
Bad	Bcl-2 antagonist of cell death
Bak	B-cell homologous antagonist/killer
Bax	Bcl-2 associated protein X
BBB	blood brain barrier
BCA	bicinchoninic acid
Bcl-2	B cell lymphocyte/leukemia-2
BH domains	Bcl-2 homology domains
BNip3	Bcl-2/adenovirus E1B 19kD-interacting protein
BNip3L	Bcl-2/adenovirus E1B 19kD-interacting protein 3-like
BSA	bovine serum albumin
Ca ²⁺	calcium ion
CAD	caspase-activated deoxyribonuclease
cAMP	cyclic AMP
CARD	caspase recruiting domain
CCA	common carotid artery
CED	cell death abnormal mutations in <i>C. elegans</i>
<i>C. elegans</i>	<i>Caenorhabditis elegans</i>

CHAPS	3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulphonic acid
CK	creatine kinase
CMXRos	chloromethyl-X-rosamine
CO ₂	carbon dioxide
CPS	cerebral protein synthesis
CsA	cyclosporin A
CSF	cerebrospinal fluid
CVA	cerebrovascular accident
Cyp D/CP-D	cyclophilin D
CYTO	cytosol
DEX	dexamethasone
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
DPX	distrene, plasticiser and xylene slide mounting medium
DTT	dithiothreitol
dATP	deoxy-adenosine triphosphate
EB	extraction buffer
ECA	external carotid artery
ECL	enhanced chemiluminescence
EDTA	ethylenediamine tetra-acetic acid
EGL-1	egg laying defective mutations in <i>C.elegans</i>
EGTA	ethylene glycol-bis (β -aminoethyl ether) <i>N,N,N,N</i> tetra-acetic acid
e/i/nNOS	endothelial / inducible / neuronal nitric oxide synthase
ELISA	enzyme-linked immunosorbent assay
ER	endoplasmic reticulum
Et-1	endothelin-1
FADH ⁺	flavin adenine dinucleotide
FCS	foetal calf serum
FITC	fluorescein isothiocyanate

g	gravity
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GFAP	glial fibrillary associated protein
GLUT-1	glucose transporter-1
h	hour
H ⁺	hydrogen ion
H ₂ O ₂	hydrogen peroxide
HBSS	Hank's Balanced Salt Solution
HeLa	human cervical carcinoma cell line
HIF	hypoxia-inducible factor
HK/HXK	hexokinase
i.c.	intracellular
<i>i.p.</i>	intraperitoneal
<i>i.v.</i>	intravenous
ICA	internal carotid artery
ICAM	intracellular adhesion molecule-1
ICE	interleukin converting enzyme
IEG	immediate early gene
Ig	immunoglobulin
Iκ-B	inhibitor of kappa light chain gene enhancer in B cells
IMM	inner mitochondrial membrane
IP ₃	inositol 1,4,4, trisphosphate
JC-1	5,5',6,6'-tetrachloro-1,1'3,3'-tetraethylbenzimidazolcarbocyanine iodide
K ⁺ Cl ⁻	potassium chloride
kDa	kilodalton
KSD	potassium serum withdrawal
MCA	middle cerebral artery
MCID	microcomputer imaging device
Mg ⁺ Cl ⁻	magnesium chloride

mg	milligram
min	minute
ml	millilitre
μ l	microlitre
mm	millimetre
μ M	micromolar
MITO	mitochondria
MTP	mitochondrial permeability transition pore
mRNA	messenger RNA
Na^+Cl^-	sodium chloride
Na_2CO_3	sodium carbonate
NAD^+	nicotinamide adenine dinucleotide
NF-AT	nuclear factor of activated T cells
NFG	nerve growth factor
$\text{NF}\kappa\text{-B}$	nuclear factor of kappa light chain gene enhancer in B cells
Nix	Nip3 interacting protein X
NMDA	<i>N</i> -methyl-D-aspartate
NO	nitric oxide
O_2	oxygen
OA	occipital artery
OGD	oxygen-glucose deprivation
OMM	outer mitochondrial membrane
PARP	poly (ADP-ribose) polymerase
PBS	phosphate buffered saline
PCD	programmed cell death
PCr	phosphocreatine
PDVF	polyvinylidene fluoride
PET	positron emission tomography
PFA	paraformaldehyde

PI3-K	phosphatidyl inositol-3-kinase
PLP	periodate-lysine-paraformaldehyde
PMF	permanent monofilament
PMNLs	polymorphonuclear leukocytes
PMSF	phenyl-methyl-sulfonyl-fluoride
RNA	ribonucleic acid
ROS	reactive oxygen species
RT-PCR	reverse transcription polymerase chain reaction
SCF	subcellular fractionation
SD	Sprague Dawley
SDS	sodium dodecyl sulphate
s.e.m.	standard error of the mean
SOD	superoxide dismutase
STA	superior thyroid artery
STS	staurosporine
TBST	Tris Buffered Saline-Tween 20 (0.5 %) buffer
TM domain	transmembrane domain
TMF	transient monofilament
TNF α	tumour necrosis factor α
tPA	tissue plasminogen activator
Tris	Tris-[hydroxymethyl]-aminomethane
TTC	2,3,5-triphenyltetrazolium chloride
TUNEL	terminal deoxynucleotidyl transferase nick end labelling
Tween 20	polyoxyethylene-sorbitan monolaurate
VDAC	voltage dependent anion channel
W	watts
Zn ²⁺	zinc
$\Delta\Psi_m$	mitochondrial transmembrane potential

*'This is the way the world ends,
Not with a bang, with a whimper'*

The Hollow Men (1925)

T.S. Elliott

CHAPTER 1: GENERAL INTRODUCTION

1.1 GENERAL INTRODUCTION

In contrast to almost every other organ, the brain has an absolute requirement for an uninterrupted supply of oxygen and glucose. The large metabolic requirement of the brain and the limited capacity for substrate storage (Benveniste *et al.* 1987) renders this organ particularly susceptible to damage during ischaemic episodes (Siesjö *et al.* 1992a). Costing the National Health Service an estimated £2.3 billion, and forecast to rise 30% by 2023, ischaemic stroke is accountable for 4.6 million deaths worldwide each year (Bonita & Beaglehole 1995). Considerable research efforts are underway to identify novel therapeutic strategies for this disease, which at present, has no effective cure. Tissue plasminogen activator (tPA), the only clinically accepted treatment, relies on thrombolysis and re-introduction of the blood supply through the occluded artery. While early reperfusion appears beneficial (Marchal *et al.* 1999), delayed onset of reperfusion has been reported to exacerbate damage both in the clinic (Kidwell *et al.* 2001) and in animal stroke studies (Cao *et al.* 2001a, Berti *et al.* 2002). Permanent vessel occlusion leads to tissue infarction that typically involves a core ischaemic lesion (or focus) and a surrounding “penumbra” which is subjected to less severe reductions in blood flow (Jacewicz *et al.* 1992, Memezawa *et al.* 1992a). These two regions differ physiologically, biochemically and in their response to neuroprotective therapies (Gingsberg *et al.* 1996, Zhang *et al.* 1995). Although damage in the core lesion appears irreversible (Siesjö *et al.* 1992a), cells in the penumbra are considered amenable to pharmacological intervention and potentially salvageable following early reperfusion (Memezawa *et al.* 1992b). In contrast to the core lesion which develops within minutes to hours and has been reported to be a primarily, passive necrotic-like event, cell damage in less severely damaged tissue reportedly progresses more slowly (days to weeks) and likely involves an active type of cell death (Linnik *et al.* 1993, MacManus *et al.* 1997, Du *et al.* 1996). Several mediators of apoptotic cell death have now been reported (Gillardon *et al.* 1996, Cao *et al.* 2001a). One such group include the pro-apoptotic Bcl-2 family, which are reported to act upstream of mitochondrial dysfunction and irreversible cell death (Kelekar *et al.* 1997). Therefore, targeting these regulatory mechanisms might be a therapeutic frontier in stroke research.

1.2 STROKE

INCIDENCE & EPIDEMIOLOGY

Focal ischaemic stroke commonly arises from occlusion of a major artery within the brain (Astrup *et al.* 1981). The concept of ischaemia is not particularly useful, and is better termed oligoemia; to describe a tissue receiving a reduced blood supply with the consequence of insufficient oxygen and substrate delivery. However, since 'ischaemia' has been used in literature to describe the condition of reduced blood flow to an organ, the term 'ischaemia' rather than 'oligoemia' will be used in this thesis. Stroke is the third leading cause of death in adults (Gorelick *et al.* 1995), with over 4 million survivors living with the debilitating consequences. It is the single largest cause of severe disability, causing 600,000 deaths per year in the UK (Office Nat Stats 1997). Cerebral ischaemia can be divided into two major categories: (i) Ischaemic stroke and (ii) Haemorrhagic stroke. Ischaemic stroke is further categorised into global ischaemia (as occurs following cardiac arrest) and focal ischaemia (as occurs following localised disruption of cerebral blood flow to the brain). Vessel occlusion may be caused by a thrombus (clot), embolism (mobilised clot from elsewhere in body) or arterial narrowing (stenosis) (Hossmann *et al.* 1982). Haemorrhagic stroke is due to a ruptured intracranial vessel. In the UK, 80% of all strokes are ischaemic in nature. Middle cerebral artery occlusion is the most common underlying cause of ischaemic stroke in humans (Nedergaard *et al.* 1988). The WHO describes stroke as "developing signs of focal (or global) disturbance of cerebral function lasting longer than 24 h with no apparent non-vascular cause (Thorvaldsen *et al.* 1995). A number of risk factors have been identified. These include non-modifiable factors (male gender, advanced age, race and hereditary which are not amenable to preventative approaches (Dyken *et al.* 1984), as well as predisposing factors (asymptomatic carotid artery stenosis, diabetes mellitus, transient ischaemic attacks (TIAs), cardiac arrest, hypertension, cigarette smoking and exercise (Gorelick *et al.* 1995).

CEREBRAL STRUCTURE

The anatomy of the human cerebral circulation was first described by Willis in 1690. The anatomy of the cerebral circulation in rats is similar to humans and is outlined in *Figure 1.2.1*. The internal carotid artery branches out into the anterior, middle and posterior cerebral arteries, which supply each hemisphere, and are connected via an azygous anterior cerebral and posterior communicating artery to form a modified Circle of Willis (Yamori *et al.* 1976). The vertebral arteries fuse to form the basilar artery that in turn supplies the posterior structures of the brain via the posterior cerebral artery. The middle cerebral artery (MCA) runs laterally over the olfactory tract and branches off to supply the

cerebral cortex. Middle lenticulostriates branch from the MCA and supply the posterior caudate putamen (striatum) and olfactory tract (Yamori *et al.* 1976). In the rat, local blood flow is similar in both the striatum and overlying cortex (Sakurada *et al.* 1978), where complete localised ischaemia is almost impossible to achieve due to numerous arterial / venous anastomoses (Scremin *et al.* 1995) which serve a protective role (del Zoppo *et al.* 2000).

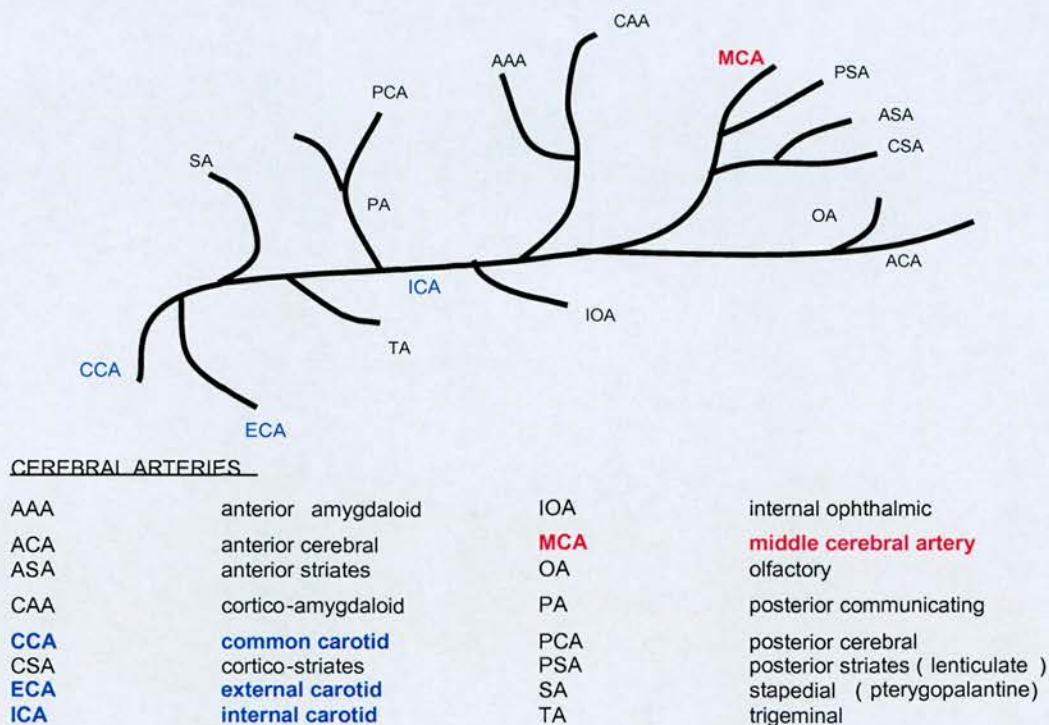


Figure 1. 2.1: Simplified diagram showing the cerebral circulation of the rat.

CEREBRAL BLOOD FLOW FUNCTION

The function of the cerebral circulation is a) to provide the necessary substrates to the brain tissue and b) to remove metabolic by-products. Three key homeostatic mechanisms serve to maintain cerebral blood flow. Firstly, relatively constant cerebral blood flow (CBF) levels are maintained over a wide range of perfusion pressures. This protects the brain against changes in systemic blood pressure, and is known as autoregulation (Fog *et al.* 1939, Harper *et al.* 1966). Secondly, the brain adapts to changes in local blood flow to accommodate fluctuations in the metabolic activity, this is referred to as flow-metabolism coupling (Roy & Sherrington 1890, Sokoloff & Kety 1960). In addition, baroreceptors in the carotid artery regulate mean arterial blood pressure. In ischaemia, autoregulation is lost. In the normal functioning brain, no energy-yielding substrate other than glucose is significantly extracted from the blood. No endogenous substrate can substitute glucose in cerebral energy metabolism. Ketone bodies are poor substitutes for glucose (Siesjö *et al.* 1978) and anaerobic metabolism of glucose to yield lactate produces little energy (Siesjö *et al.* 1984). Brain injury following focal

ischaemia is invariably the consequence of an inadequate supply of oxygen and metabolic substrates to the brain tissue (Fieschi *et al.* 1978), and as such, may be considered a disease of substrate depletion. Astrup (1977) first described the concept of viability thresholds to explain tissue damage during cerebral ischaemia (Figure 1.2.2), although, absolute thresholds vary between species (Hossmann *et al.* 1994). Transient increases in glucose utilisation during reduced CBF are rapidly replaced by a sharp decline during anaerobic respiration (Hossmann *et al.* 1994). A further decline in CBF is accompanied by reductions in intracellular adenosine triphosphate (ATP) and phosphocreatine (PCr). Loss of electrical excitability occurs when cerebral blood flow is below one-third normal levels, whereas membrane failure and loss of ion homeostasis occur when CBF is 25% pre-ischaemic values (Siesjö *et al.* 1992a). At this level, neuronal cell death occurs within minutes (Sweeney *et al.* 1995). In the rat CBF below $25\text{ml}\cdot 100\text{g}^{-1}\cdot \text{min}^{-1}$ produces well-demarcated tissue damage. This threshold is higher than for larger species, likely explained by the higher neuronal density in rat brain (Hossmann *et al.* 1994, Back *et al.* 1998).

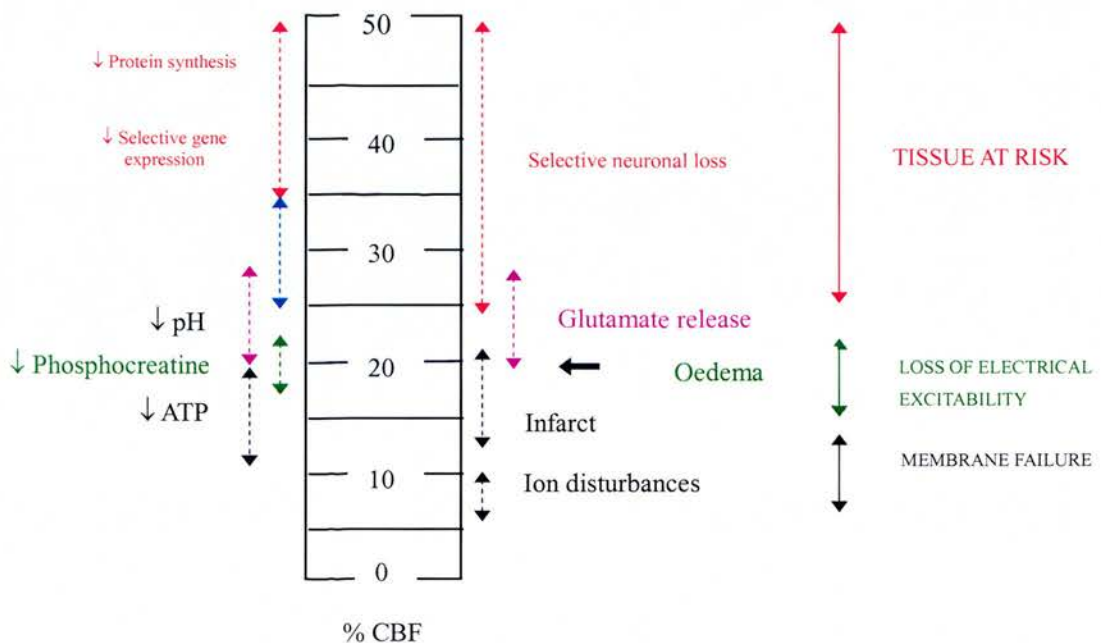


Figure 1.2.2: Viability thresholds of cerebral ischaemia (compiled from Hossmann, 1994; Siesjö, 1984; Siesjö 1992a)

CONCEPTS OF CORE, PENUMBRA & REPERFUSION INJURY

The concept of a core and penumbra was first proposed by Symon in 1974. The core was defined as the central lesion of mainly necrotic cells exposed to severe reductions in cerebral blood flow. Hossmann (1994) coined the term “penumbra” as it resembled a partially illuminated area around the complete shadow of the moon during a full eclipse. As such, the “penumbra” was defined as tissue

containing electrically silent, structurally intact and essentially viable neurones, which lie between areas of the core infarct and normal brain tissue (Astrup *et al.* 1977, Symon *et al.* 1974, Symon *et al.* 1977). Astrup *et al.* (1981) redefined the penumbra as brain tissue perfused at a level between thresholds for functional impairment and loss of morphological integrity, with the capacity to recover if perfusion is re-established. The concept of an ischaemic “penumbra” was thus redefined to denote damaged, but potentially salvageable tissue (Siesjö *et al.* 1992a, b). The penumbra has also been described as “an evolving zone of bioenergetic upheaval” (Gingsberg & Pulsinelli 1994): The majority of tissue damage within the core lesion is reported within the first few hours compared to the penumbra which continues to deteriorate at much later time points (Garcia & Kamijyo 1974, Heiss *et al.* 1983, Mies *et al.* 1983), where neuronal survival might depend on both the severity and duration of vessel occlusion (Hallenbeck & Dutka 1990). Lesion expansion has been attributed to recruitment of cells into the core lesion until well-perfused tissue is encountered, or to the coalescence of islands of necrotic cells (Siesjö *et al.* 1992a) (*Figure 1.2.2*) mediated by calcium overload (Siesjö *et al.* 1992b, Siesjö *et al.* 1999) and/or peri-infarct spreading depression-like depolarisations extending from the margins of the lesion (Nedergaard & Hansen 1988). Over the past 30 years the term ‘penumbra’ has been defined by electrophysiological, pharmacological, histological, imaging and biochemical methods. However, for the purpose of this thesis the term ‘penumbra’ will be used simply to describe compromised tissue surrounding the core ischaemic lesion.

REPERFUSION – SAINT OR SINNER?

Reversal of vessel occlusion (re-canalisation or reperfusion) can occur in humans, either spontaneously or following thrombolytic treatment. Human positron emission tomography (PET) studies suggest that reperfusion is beneficial to the prognosis of stroke, and supports the use of early thrombolysis (Marchal *et al.* 1999). However, re-canalisation is a complex and controversial issue with regards to stroke outcome, where it has been implicated in both (i) cessation of cell death process with return of substrates and (ii) exacerbation of damage in already compromised tissue, after reintroduction of blood supply and possible pro-inflammatory mediators (Feuerstein *et al.* 1994, Kuroda & Siesjö 1997). Thus, the molecular events underlying ischaemia-induced brain damage are influenced both by whether the occlusion is reversed or permanent, and by the severity of the ischaemic insult under investigation. Although considerable progress has been made in identifying processes that contribute to tissue damage, the mechanisms remain incompletely defined, as is the issue of reperfusion injury.

MODELS OF CEREBRAL ISCHAEMIA

The use of young animals to model the clinical condition of stroke, a disease of mid-late life with often co-existing complications, has been much debated (Millikan 1992). Animal models have however proved essential in characterising particular aspects of the disease process and in neuroprotection studies. Standardised animal strains, experimental approaches and reproducible experimental models of ischaemia have proved critical in understanding the pathophysiology of stroke (Hsu *et al.* 1993). It is now known that several physiological variables (blood pressure, brain / body temperature and blood gases) have adverse effects on the neurological outcome following an ischaemic insult, and should, where possible should be monitored and kept within physiological limits (Oloff *et al.* 1995). Cause, severity, location of insult and predisposition of existing systemic diseases are uncontrollable variables in stroke (Hsu 1993). Therefore, large sample sizes are needed to account for such variables in human studies (MacRae *et al.* 1992). Consequently, attempts to dissect the pathophysiology of human stroke have proved difficult. Histological, biochemical and physiological investigation requires invasive surgery and direct access to the brain (Gingsberg & Busto 1989). To establish such data in humans would not only require patient compliance, but would be difficult to support ethically.

RODENTS – SUITABLE SPECIES TO MODEL ISCHAEMIA

Animal models of middle cerebral artery (MCA) occlusion were first developed during the early 1930s in primates (Peterson *et al.* 1937). This technique has now been adapted and refined in other species ranging from rodents to cats and large non-human-primates (Gingsberg & Busto 1989). Rodent models are now most widely used for several reasons, including low cost and maintenance, inbreeding-induced homogeneity, similar cranial circulation to man, small brain size suitable for fixation and histological assessment, and ethical acceptability (Gingsberg & Busto 1989). Moreover, advances in gene technology support the need at present for rodent models of focal cerebral ischaemia (Chabrier *et al.* 1999). Although continual refinement of animal models is critical to our understanding of particular aspects of the disease pathway. It is however important to note that no stroke model exactly reproduces the human condition presented to the clinic.

MIDDLE CEREBRAL ARTERY OCCLUSION

Numerous reproducible models of focal ischaemia have been developed (Gorelick *et al.* 2000) and the advantages and disadvantages of each model are outlined in Table 1.2.1. For many years, middle cerebral artery occlusion in the rat has been the gold standard of ischaemic stroke models. This originally involved surgical exposure of the overlying skull, craniotomy followed by diathermy of the vessel and unsuitable for recovery studies (Tamura *et al.* 1981). However, this approach is technically very demanding and associated with post-surgical feeding problems. An alternative method for occluding the MCA; the intraluminal monofilament model, was used within F.I.N.E. laboratories and

was the most commonly reported model in literature, at the time of study. Therefore, the intraluminal monofilament model of MCA occlusion was the model of used in this thesis.

INTRALUMINAL FILAMENT MCA OCCLUSION

Koizumi *et al.* (1986) first described a model of focal cerebral ischaemia that allows investigation of both permanent and transient (with reperfusion) ischaemia avoiding craniotomy (*Figure 1.2.3*). Introduction of a monofilament suture from the external to internal carotid arteries, with advancement of the suture beyond the origin of the MCA provides a reproducible pattern of cortical and striatal damage (see Chapters 2, -6 and -7). However, insertion of the suture might exacerbate damage incurred during reperfusion (Macrae 1992, Schmid-Elsaesser *et al.* 1998). Zea Longa and colleagues (1989) refined this technique by blunting and advancing the monofilament towards the proximal anterior cerebral artery. In addition, all branches of the external carotid artery (ECA) and extracranial branches to the internal carotid artery (ICA) were occluded to minimise blood loss. However, the Koizumi model produced a more profound reduction in cerebral blood flow with increased reproducibility (Laing *et al.* 1993). A further refinement to the Koizumi model involved blunting the monofilament and coating it with poly-L-lysine (polycationic amino acid). This coating has been reported to encourage adhesion of the monofilament to the endothelium, and to improve reproducibility of lesion volume and location (Belayev *et al.* 1996). This monofilament model, with slight variations, is the most widely used experimental model since it is highly reproducible, with comparatively short surgical procedure, adequate temperature regulation, low mortality rate, minimal post-operative complications and ability to control reperfusion following filament withdrawal. As such, the intraluminal filament model was used in this thesis.

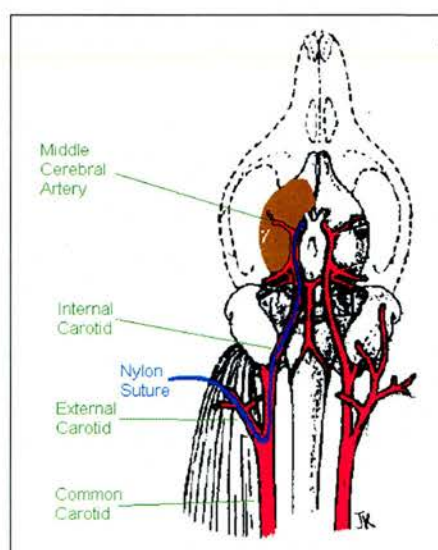


Figure 1.2.3: Monofilament occlusion model of middle cerebral artery occlusion (Koizumi et al., 1986). See text for details

Table 1.2.1: Models of middle cerebral artery occlusion in the rat

METHOD OF OCCLUSION	ADVANTAGES	DISADVANTAGES	REFERENCE
Electrocoagulation/ligation/clipping of the proximal MCA via sub-temporal craniotomy	Reproducible infarct Volumes representative of large human lesions Reperfusion possible (with clips)	Thermal damage and desiccation due to sub-temporal craniotomy Mechanical damage to vessel Possible post-surgical feeding problems Anaesthesia required	Tamura <i>et al.</i> 1981 Shigeno <i>et al.</i> 1985
Intraluminal monofilament	Relatively easy surgical procedure No need for craniotomy and associated problems No post surgery feeding difficulties Reperfusion can be achieved and animal consciousness recovered	Damage to endothelium by insertion of monofilament Risk of puncturing the vessel Occlusion of ECA and its branches and pterygopalantine artery which increases infarct volume Anaesthesia required for insertion of monofilament although not necessarily for reperfusion	Koizumi <i>et al.</i> 1986 Zea Longa <i>et al.</i> 1989
Abluminal application of Endothelin (Et) via sub-temporal craniotomy	No mechanical damage to vessel	Sub-temporal craniotomy and associated problems Anaesthesia required Possible interaction of potential neuroprotectants with Et and receptors Uncontrolled reversal of occlusion	Robinson <i>et al.</i> 1990
Stereotaxic application of Et isozeptides via dorsal craniotomy	No mechanical damage to vessel Indwelling cannula can be used which allows administration of Et to conscious animals	Craniotomy required although not as demanding as sub-temporal approach Needle tract damage Possible interaction of potential neuroprotectants with Et and receptors	Sharkey & Butcher 1995 Henshall <i>et al.</i> 1999
Photochemically induced thromboembolism	No craniotomy required (depends on thickness of the skull) Site of occlusion chosen by irradiating desired target area	Possibility of thermal damage by the light source Generation of free radicals which affect integrity of BBB Instability of clot and subsequent multiple infraction may occur Inability to control reperfusion	Watson <i>et al.</i> 1985
Introduction of autologous/heterologous emboli /microspheres/ macrospheres	Minimal surgical intervention Embolic model useful for assessing thrombolytic treatments	Instability of clot and subsequent multiple infarction may occur Variation in infarct volume and location Inability to control reperfusion	Papadopoulos <i>et al.</i> 1987 Kudo <i>et al.</i> 1982 De Visscher <i>et al.</i> 2003

PATHOLOGICAL CONSEQUENCES OF FOCAL ISCHAEMIA

Occlusion of a major vessel within the brain leads to cell death via a number of inter-related pathways, which include impaired energy production, calcium overload, glutamate-induced excitotoxicity, lipid peroxidation, mitochondrial dysfunction, altered protein synthesis and apoptosis (Table 1.2.2, Figure 1.2.4).

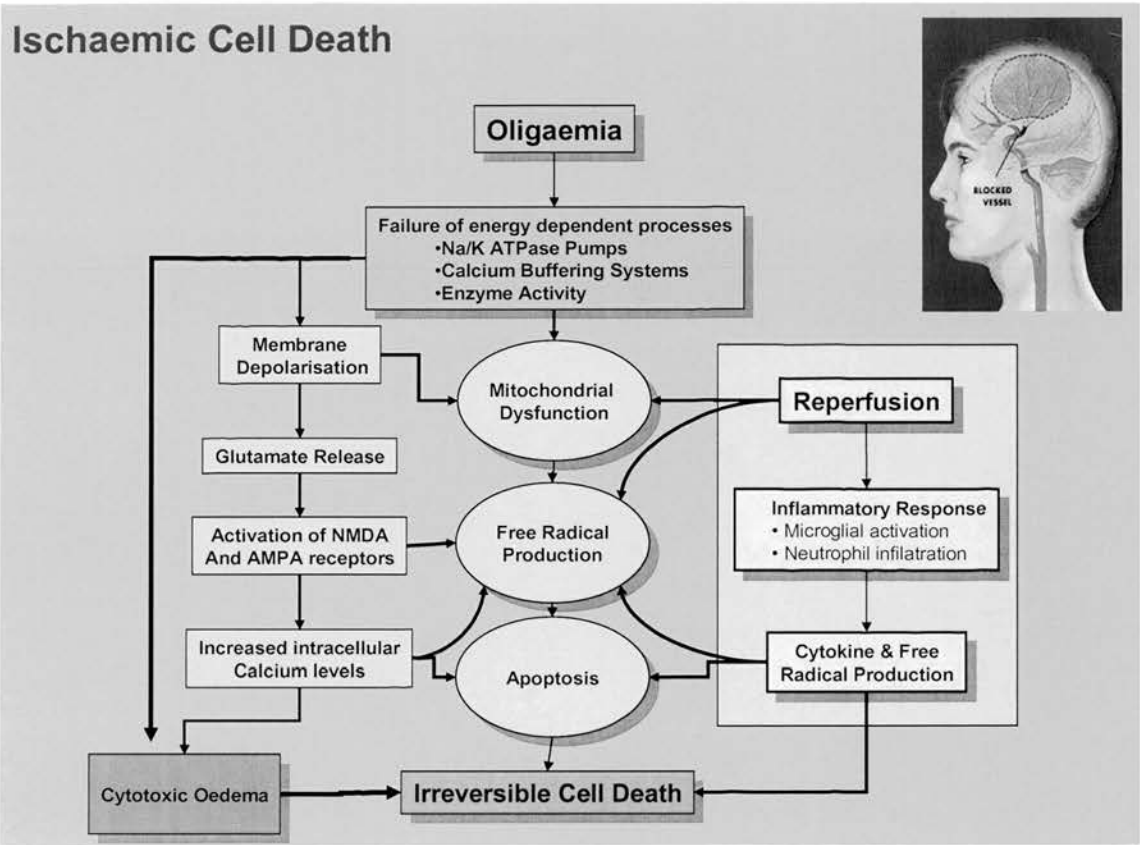


Figure 1.2.4: The Ischaemic Cascade. (See text for details).

Rapid ATP depletion during ischaemia leads to intracellular acidification and inhibition of ATP dependent pumps, including Na^+/K^+ ATPase (Wallimann 1992). Consequently, sodium enters the cell down its concentration gradient (with obligated chloride and water), which leads to anoxic depolarisation (Graham *et al.* 1994) and oedema (Betz *et al.* 1994). Anoxic depolarisation is responsible for excessive increases in intracellular calcium (Kristian & Siesjö 1998, Krupinski *et al.* 2000) and extracellular glutamate (Choi *et al.* 1988, Hossmann *et al.* 1994); both of which have been implicated as the major mediators of both necrotic and apoptotic cell death observed following focal cerebral ischaemia.

Table 1.2.2: Summary of the pathophysiology of focal cerebral ischaemia..

ISCHAEMIC MEDIATOR	PATHOPHYSIOLOGICAL CHANGES	REFERENCE
ATP DEPLETION & ANOXIC DEPOLARISATION	Na ⁺ /K ⁺ ATPase pump failure Membrane depolarisation, Na ⁺ Cl ⁻ and H ₂ O influx, swelling & lysis	Siesjö <i>et al.</i> , 1988 Siesjö, 1992a
ACIDOSIS & OEDEMA	Na ⁺ ion influx and blood brain barrier disruption promote oedema	Plum <i>et al.</i> , 1983 Hatashita & Hoff, 1990 Siesjö, 1992a Betz <i>et al.</i> , 1994
CALCIUM & GLUTAMATE TOXICITY	↑ levels of intracellular calcium Activation of Ca ²⁺ -dependent proteolytic enzymes, second messengers, generation of reactive oxygen species (ROS) Activation of receptor- & voltage operated glutamate receptors Massive increase in extracellular glutamate Excitotoxicity	Choi <i>et al.</i> 1988 Siesjö <i>et al.</i> 1989 Hossmann <i>et al.</i> , 1994 Lipton <i>et al.</i> 1999 Obrenovitch <i>et al.</i> , 2000
REACTIVE OXYGEN SPECIES	Generated in mitochondria Excessive Ca ²⁺ _{IC} during ischaemia promotes excessive ROS production Lipid peroxidation, membrane damage, protein & DNA damage Free radical scavengers are neuroprotective	Siesjö, 1992b Yang <i>et al.</i> , 1994 Chan <i>et al.</i> , 1994 Kuroda & Siesjö, 1997
NITRIC OXIDE & NITRIC OXIDE SYNTHASE	Neuromodulator – dual functions NOS isoforms activated during ischaemia eNOS beneficial, nNOS & iNOS detrimental NOS inhibitors reduce infarct size	Martin <i>et al.</i> , 1994 Katsura <i>et al.</i> , 1994 Colasanti & Suzuki, 2000
INFLAMMATION	Endogenous response in parenchyma & endothelia Exogenous response in microvasculature ↑ TNF-α, ↑ IL-1β; cytokine release Role in ischaemia argued	Rothwell <i>et al.</i> 1995 Hallenbeck <i>et al.</i> , 1997 Barone & Feuerstein 1999 Del Zoppo <i>et al.</i> , 2000
GENE EXPRESSION & PROTEIN SYNTHESIS	↓ Protein synthesis (translation) Yet, ↑ mRNA & protein expression of numerous regulatory molecules (e.g. immediate early genes, heat shock proteins, transcription factors) Upregulation of transcription factors p53 & Hypoxia Inducible Factor-1α (HIF-1α)	An <i>et al.</i> , 1993 Akins <i>et al.</i> , 1996 Johansson <i>et al.</i> , 2000 Wang <i>et al.</i> , 2002
APOPTOSIS	Ca ²⁺ -mediated endonuclease activation & nuclear DNA changes Activation of regulators of apoptosis, including caspases & Bcl-2 family HIF-1α & p53-mediated Bcl-2 family activation	Linnik <i>et al.</i> , 1993 MacManus <i>et al.</i> , 1997 Du <i>et al.</i> , 1996 Nicotera & Lipton, 1999 Banasiak 2000 Hengartner <i>et al.</i> , 2000

1.3 MITOCHONDRIAL CONTROL OF CELL FATE

The term mitochondria (mitos [thread] and chondros [granule]) was introduced by Brenda and colleagues in 1898. However, the presence of these granules, or bioblasts, had been known for 40 years previously (Kolliker *et al.* 1856) and their preliminary structure and function investigated (Altman *et al.* 1890). However, it was not until the mid 1940's that Hogeboom and colleagues identified the mitochondria as the major site of energy metabolism. Central to mitochondrial function is control over oxidative phosphorylation and energy production (Lill *et al.* 1996). Less well known perhaps is their role in maintaining ion homeostasis and acting as buffers for calcium and hydrogen ions, neutralizing reactive oxygen species produced during oxidative phosphorylation and control over both necrotic and apoptotic cell death mechanisms.

Mitochondria play a critical role in cell life and death. They provide for the bioenergetic needs of the cell, control calcium homeostasis and handle the most toxic of molecules, oxygen, without harming the cell itself as well as sequestering an arsenal of death-promoting molecules. It is now understood that during apoptosis, mitochondria release several apoptotic mediators into the cytosol (for reviews see Green & Reed 1998, Kroemer & Reed 2000). In order to understand the role of mitochondria in the context of apoptosis, it is important to review the structural arrangements of this organelle. Mitochondria are organelles with two well-defined compartments: the matrix, surrounded by the inner mitochondrial membrane (IMM) and the intermembrane space, surrounded by the outer mitochondrial membrane (OMM). The outer and inner membrane are in close opposition at a number of sites and form the contact points believed to contain the protein import and ADP/ATP exchange machinery responsible for transport of molecules between the matrix and cytoplasm (Perkins *et al.* 1997). The IMM contains various molecules, including ATP synthase, electron transport chain and adenine nucleotide translocator (ANT). Under physiological conditions these molecules allow the respiratory chain to create an electrical gradient (membrane potential). The OMM contains a voltage-dependent anion channel (VDAC). The intermembrane space contains apoptotic mediators such as cytochrome c, certain pro-caspases, adenylate kinase 2 and apoptosis-inducing factor (Manella *et al.* 1994). Apoptosis induces permeabilisation of the OMM, resulting in the release of these molecules, whereas IMM permeabilisation leads to changes in mitochondrial membrane potential and has been implicated under more necrotic-like conditions; markers of both have been reported during focal cerebral ischaemia (Kristian & Siesjö 1998, Siesjö *et al.* 1999).

ATP PRODUCTION

Mitochondria provide cells with energy in the form of adenosine triphosphate (ATP). The number of mitochondria per cell is roughly related to cell energy demands. In major mammalian tissues, 80 to 90% of ATP is generated by mitochondria in the process of oxidative phosphorylation. Sugars (e.g. glucose) or fatty acids provide fuel for ATP production, which are broken down into two-carbon units, acetyl-CoA, which then enter the Krebs cycle within the mitochondrial matrix, and become oxidized, to water and carbon dioxide (CO_2). The resulting high-energy electrons are transferred along a chain of carriers that are situated in the inner mitochondrial membrane. Electrons (e^-) are transferred to NAD^+ and FADH^+ , which produce reducing equivalents, NADH and FADH_2 that become electron donors during oxidative phosphorylation (Nicholls *et al.* 1992). Cytochrome c serves as a one-electron shuttle between complexes III and IV, where the electrons are transferred to oxygen. Leakage of protons from the cytoplasm back in towards the mitochondria drives ATP production (Figure 1.3.1).

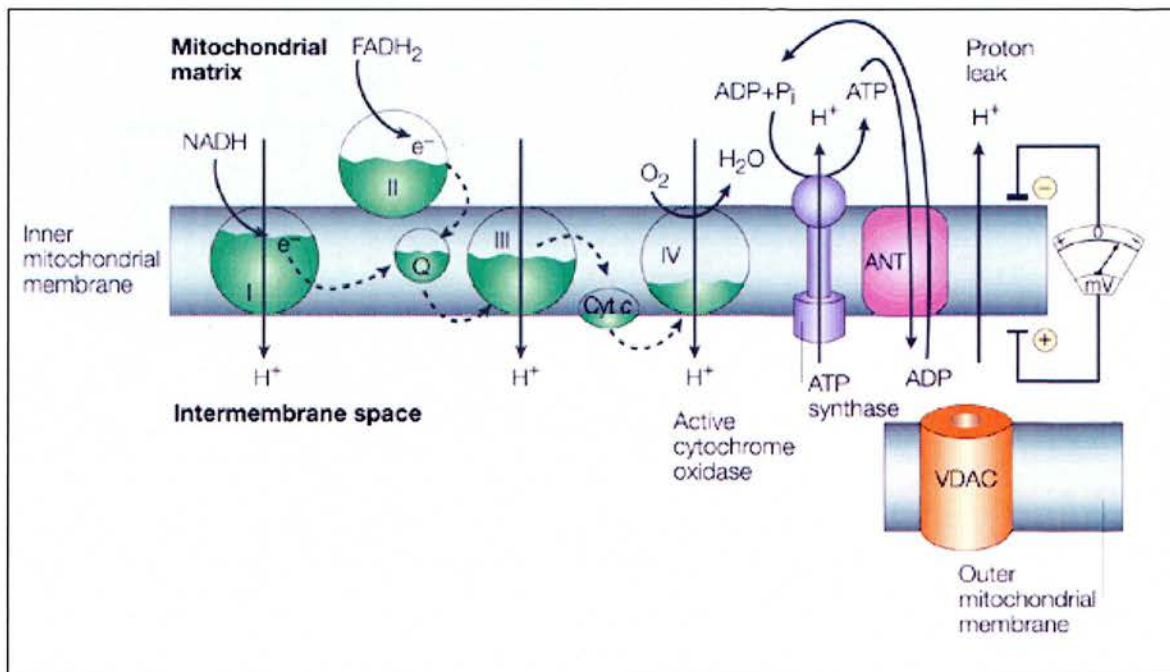


Figure 1.3.1: Schematic diagram of oxidative phosphorylation and ATP generation in the mitochondria (Adams 2002. Diagram not modified).

As electrons (e^-) are passed along the electron transport chain, protons (H^+) are pumped out into the cytosol, which generates an electrochemical gradient ($\Delta\Psi$) across the inner mitochondrial membrane and provides the force for ATP production within the mitochondria.

GENERATION OF FREE RADICALS & CALCIUM BUFFERING

Mitochondria produce reactive oxygen species (ROS) as a normal by-product of oxidative phosphorylation. During conditions of mitochondrial dysfunction increased superoxide levels cause oxidation of other molecules within the cell, in turn leading to DNA mutations and irreparable damage to proteins. It has been demonstrated that prolonged increases in nitric oxide (NO) as occurs during focal ischaemia, will eventually result in $\Delta\Psi_m$ collapse, ATP depletion, and ultimately cell death (Beltran *et al.* 2000, Almeida *et al.* 2001). Mitochondrial control of calcium homeostasis appears intricately involved in the cell's response to apoptotic signals. Increases in cytosolic calcium are often observed in response to chemicals such as staurosporine (Kruman *et al.* 1998), fas (Chien *et al.* 1999), hydrogen peroxide (Creagh *et al.* 2000) or thapsigargin, an inhibitor of the sarcoplasmic/endoplasmic reticulum Ca^{2+} -ATPase (Marin *et al.* 1996). To protect the cell, mitochondria accumulate calcium, but at high levels this decreases the membrane potential (Zhu *et al.* 1999), leading to increased ROS production, membrane depolarisation, cytochrome c release and cell death (Kruman *et al.* 1998).

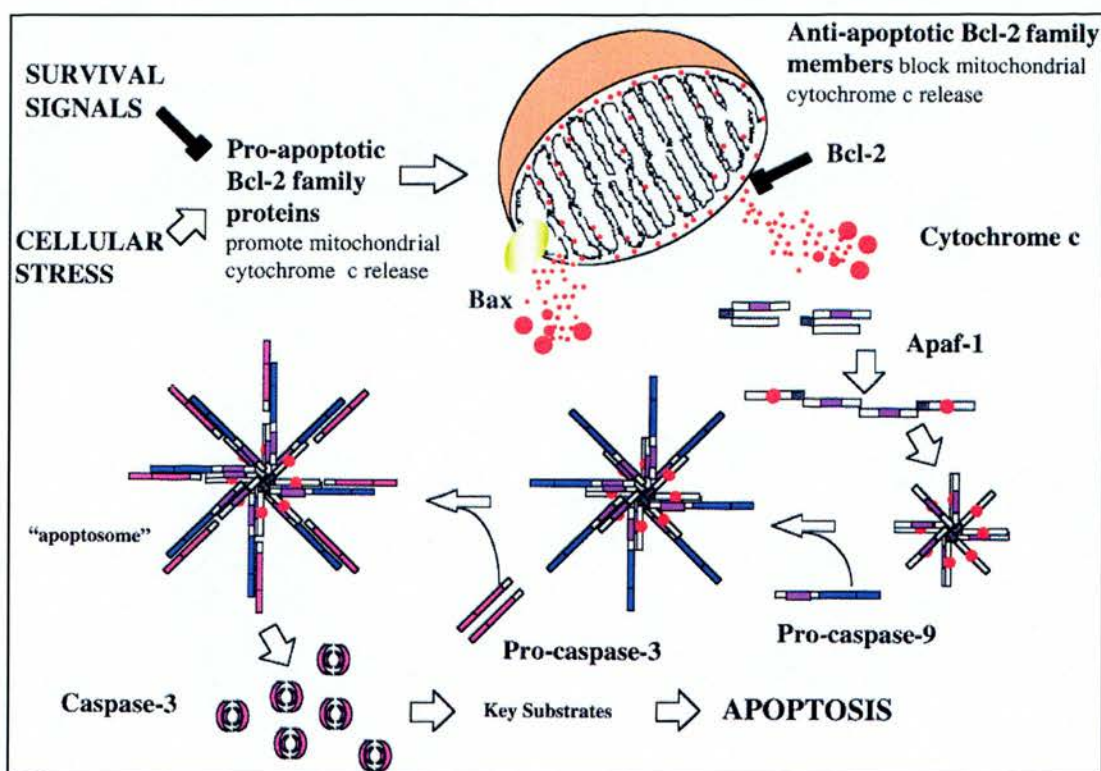
RELEASE OF APOPTOTIC MEDIATORS

Several key findings support a critical role for the mitochondria in apoptotic cell death: (i) enriched mitochondrial fractions were reported necessary for the induction of apoptotic cell death in cell-free systems (Newmeyer *et al.* 1994), (ii) Bcl-2, an anti-apoptotic protein, was localised mainly to the outer mitochondrial membrane (Nakai *et al.* 1993, Nguyen *et al.* 1993), and (iii) apoptosis was often accompanied by $\Delta\Psi_m$ loss (Zamzami *et al.* 1995). The observation that cytochrome c was involved in caspase activation (Liu *et al.* 1996, Zhivotovsky *et al.* 1998), and the demonstration that pro-apoptotic Bcl-2 family members promote mitochondrial release of cytochrome c release (Luo 1998, Li 1998, Jurgensmeier 1998), an event that is counteracted by anti-apoptotic Bcl-2 family members (Kluck *et al.* 1997, Yang *et al.* 1997, Vander Heiden *et al.* 1997), firmly established the mitochondrion as a central regulator of cell life and death (*see section 1.4; Apoptotic Cell Death*). It is now well established that mitochondria play a critical role in the regulation of apoptosis where they act as a receiving platform, integrating a range of apoptotic stimuli and relating these to the appropriate downstream signalling cascades.

APOPTOSOME FORMATION

Upon reception of a pro-apoptotic signal, mitochondria release a number of apoptogenic proteins. The first such protein to be identified was cytochrome c. Cytochrome c-dependent apoptosome formation

and subsequent mitochondrial-mediated apoptotic cell death are outlined in *Figure 1.3.2*. However, whether such an ATP-consuming event might occur under conditions of ATP depletion, such as focal cerebral ischaemia, remains a subject of much debate (Li *et al.* 1997, Liu *et al.* 1996, Zou *et al.* 1999, Du *et al.* 2000).



*Figure 1.3.2: Mitochondrial-mediated cell death cascade; formation of the apoptosome (figure from Zimmermann *et al.* 2001).*

ATP-mediated conformational change in APAF-1 allows cytochrome c to bind. APAF-1 oligomerisation-induced caspase-recruitment domain (CARD) exposure recruits procaspase-9 via CARD-CARD interactions, which in turn, forms the apoptosome. Procaspase-9 oligomerises, resulting in the autocatalysation into the cleaved and active protein and subsequent activation of downstream effector caspases, such as caspases-3 and ultimately apoptotic cell death.

MECHANISMS OF CYTOCHROME C RELEASE

One of the early events in the apoptotic cell death cascade is the release of cytochrome c from mitochondria (Liu 1996, Kluck *et al.* 1997, Reed *et al.* 1997, Martinou *et al.* 2000). This commits the cell to die by either a rapid apoptotic mechanism involving the apoptosome, or by a slower necrotic-like process due to collapse of electron transport, as occurs following cytochrome c depletion, promoting ROS generation and ATP depletion. An area of intense scrutiny is the mechanism of cytochrome c release: the central event for the induction of mitochondrial-mediated apoptotic cell death. Several models have been proposed and experimental evidence in support of each of them has

been published. However, various aspects of these models are inconsistent and await clarification. The formation of autonomous Bax-like channels (possibly involving other family members) and non-specific permeability transition pore (PTP)-mediated matrix swelling and OMM rupture are two prevailing mechanisms of cytochrome c release during apoptosis. According to some authors (Vander Heiden *et al.* 1997, Petit *et al.* 1998) but not all (Jurgensmeier *et al.* 1998, Doran & Halestrap 2000), mitochondrial swelling and OMM rupture/pore formation precede cytochrome c release.

MITOCHONDRIAL DYSFUNCTION IN STROKE

The consequences of stroke have been described essentially as a disease of mitochondrial dysfunction, where these organelles have been implicated in both apoptotic and necrotic cell death mechanisms in the ischaemic brain (Halestrap *et al.* 2000). For example, transient (with reperfusion) focal ischaemia has been associated with an initial decline in mitochondrial respiratory function as well as secondary mitochondrial damage during reperfusion where partial recovery of mitochondrial function has been implicated in exacerbation of ischaemia damage (Nakai *et al.* 1997).

Several lines of evidence support a role for mitochondrial dysfunction in ischaemic cell death. These include: (i) early release of cytochrome c from the mitochondria during permanent and transient ischaemia (Fujimura *et al.* 1999), (ii) increased free radical production and neuroprotection offered by free radical scavengers, particularly in transient models of ischaemia (Fujimura *et al.* 2000), (iii) increased levels of free nitrotyrosine (metabolic product of the free radical, peroxynitrite (Brown *et al.* 1999, Murphy *et al.* 1999) and (iv) increased hydroxyl radicals in the ischaemic brain (Morimoto *et al.* 1996, Solenski *et al.* 1997). Folbergrova *et al.* (1992, 1995) provided the most detailed characterisation of early metabolic responses following MCA occlusion. These and other studies, reported an almost immediate reduction in ATP levels to 30% pre-ischaemic values in the core, with less reductions in the peri-infarct regions (Selman *et al.* 1987, Welsh *et al.* 1991, Sun *et al.* 1995, Hata *et al.* 2000a). Despite return of high energy-yielding substrates upon reperfusion, ATP re-synthesis may remain compromised (Krause & Tiffany 1993). Some studies have however reported a rapid and almost complete return of ATP in the penumbra, with at least partial restoration of ATP levels in the core lesion, upon recirculation (Hata *et al.* 2000b).

1.4 APOPTOTIC CELL DEATH

APOPTOSIS OR NECROSIS: DEFINITIONS & CHARACTERISATION

Cells die by two primary processes: (i) necrosis, which involves the release of intracellular proteases and induces an inflammatory response, or (ii) apoptosis, where the cell remnants quietly disappear as they are phagocytosed by neighbouring cells.

Cell death by necrosis usually follows major pathological acute injury, or trauma, such as hypoxia, hyperthermia, viral invasion, exposure to various exogenous toxins, or attack by complement, causing acute loss of cellular regulation and function (Cotman & Anderson 1995). Necrosis may reflect a passive cellular response, characterised by early mitochondrial swelling and dysfunction, marginal clumping of chromatin (karyorrhexis), dilatation of the endoplasmic reticulum (ER) and ribosome dispersal. Subsequently, cells undergo nuclear, mitochondrial and plasma membrane disruption, ribosome and lysosome dissolution, nuclear swelling with loss of homeostasis, cell swelling and rupture. Loss of basophilia produces a 'ghost-like' appearance. The loss of cell membrane integrity with concomitant release of cell contents, including proteases and lysozymes, induces an inflammatory response where cytokines are released by macrophages to remove cell debris and begin the repair process (Wyllie *et al.* 1980, Kerr *et al.* 1972).

In contrast, programmed cell death (PCD) is an evolutionarily conserved form of cell suicide, which is a necessary component of tissue homeostasis and developmental processes, shaping and refining of the nervous system, as well as a physiologic response to irreparable cell injury (Krammer *et al.* 1999, Vaux *et al.* 1999). Unlike pan-cellular death as occurs during necrosis, apoptosis is generally considered to affect single cells (Kerr *et al.* 1972, Wyllie *et al.* 1980). Dysregulated apoptosis, a form of PCD, has been attributed to underlie some cases of cancer, stroke as well as several neurodegenerative disorders including spinal cord injury, autoimmunity, AIDS, Parkinson's disease and Huntington's disease, but perhaps less convincingly Alzheimer's disease (Robertson *et al.* 2000). Cells undergoing apoptosis are characterised by a set of morphologic features (Kerr *et al.* 1972). These include membrane blebbing (zeiosis), chromatin condensation, nuclear fragmentation, loss of adhesion and rounding (in adherent cells) and cell shrinkage. In addition, mitochondria and ribosomes retain their gross structure and at least partial function. Biochemical features associated with apoptosis include internucleosomal DNA cleavage, leading to an oligonucleosomal "ladder" (Cohen *et al.* 1994); phosphatidylserine (PS) externalisation on the plasma membrane (Martin *et al.* 1995); and proteolytic cleavage of a number of intracellular substrates (Martin & Green 1995). In the termination

phase, membrane-enclosed vesicles, the small remnants of the cell (apoptotic bodies), are engulfed by phagocytes (Krammer *et al.* 1999), which prevents an inflammatory response. The hallmark of the end stage of apoptosis is endonuclease cleavage of DNA in the internucleosomal linker regions, yielding 180 base-pair fragments. Separation of these fragments by agarose gel electrophoresis reveals the characteristic DNA ladder pattern of apoptosis; this is in contrast to complete DNA degradation and a smear pattern observed with cell necrosis. Apoptosis, at least in some models, appears to be an active energy-dependent process requiring ATP, RNA and proteins synthesis. It is estimated that either too much or too little cell death contributes to half of the main medical illnesses to date, for which adequate therapy or prevention is lacking (Thompson *et al.* 1995, Reed *et al.* 2000). Consequently, great interest has emerged in devising therapeutic strategies for modulating the key molecules that make these life-or-death decisions.

At the molecular level, the cell death program consists of three main phases: initiation, execution, and the degradation, or termination of apoptosis. First the cell 'senses' or receives a death stimulus. Depending on the specific trigger and particular cell type, any one of many different internal signalling cascades may be initiated during the induction phase of apoptosis. However, these pathways converge on one or two points in the execution or commitment phase of the program, at the level of the mitochondria, before the external manifestations of the suicidal or degradation phase become apparent (Green & Kroemer 1998).

APOPTOSIS – EVOLUTIONARY CONSERVED CELL DEATH MECHANISM

Seminal studies in *C. elegans* have provided a genetic framework for the death program with the identification of specific death genes, including *ced-3*, *ced-4*, *ced-9*, and *egl-1*, conserved across different species (Metzstein *et al.* 1998, Liu *et al.* 1999). In this cell-death pathway, CED-3 (*C. elegans* defective) functions as the final executioner of apoptosis. CED-3 is activated by CED-4, which is normally inhibited by CED-9 (Hengartner *et al.* 1992, del Peso *et al.* 2000). A family of cysteine aspartyl-specific proteases (caspases) represent the mammalian ortholog for CED-3 (Cryns *et al.* 1998); reported essential for all 131 programmed cell deaths that occur during hermaphrodite development. Caspase-3 appears critical in most mammalian forms of apoptotic cell death. Apoptotic protease activating factor (APAF-1) is the mammalian counterpart of CED-4 (Zou *et al.* 1997), a required co-factor for caspase activation in most models of apoptosis (Li *et al.* 1997, Zou *et al.* 1997) and mammalian orthologs of CED-9 comprise the Bcl-2 family (Kluck *et al.* 1997, Yang *et al.* 1997, Adams *et al.* 1998).

THE BCL-2 FAMILY

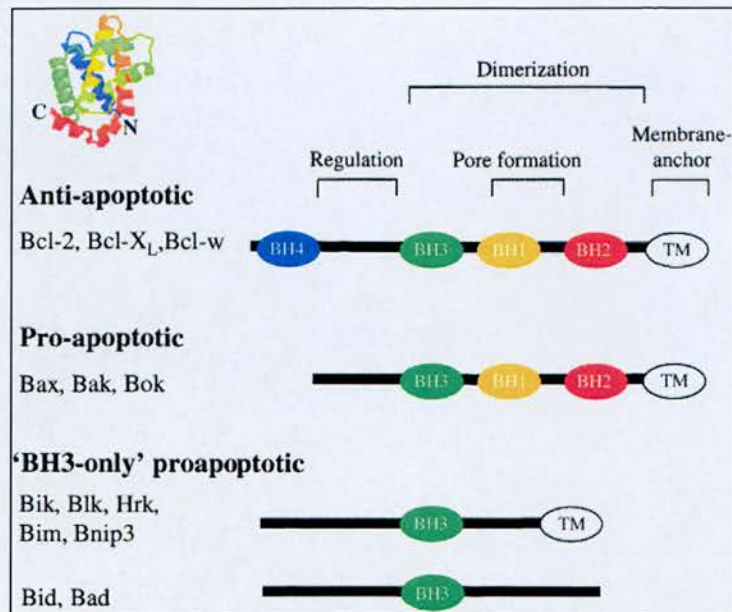


Figure 1.4.1: Classification of the Bcl-2 protein family. (Picture from Zimmermann *et al.* 2001).

Anti- and pro-apoptotic Bcl-2 family proteins are categorised by structure and function. Bcl-2-like members contain four Bcl-2 homology (BH) domains and a transmembrane (TM) domain which anchors these proteins to intracellular membranes. (See quaternary structure above). In contrast, pro-apoptotic members are subdivided: multidomain (e.g. Bax), BH3-only (with TM domain, e.g. Bnip3, Nix) and BH3-only (without TM domain, e.g. Bad).

Two major apoptosis pathways have been identified: the mitochondrial 'intrinsic' pathway and the death receptor 'extrinsic' pathway (Ashkenazi & Dixit 1998, Gupta *et al.* 2000a). However, recent evidence suggests that, at least in some cell types, these two pathways may not be mutually exclusive (Gupka *et al.* 2000b). Bcl-2 family members play key roles in the regulation of cell death and appear to govern the decision to die at multiple checkpoints, acting both at the level of the mitochondria as well as pre- and post-mitochondrial stages (Kroemer *et al.* 1997). Some 25 Bcl-2 family members have been identified, many of which homo- and/or heterodimerise with each other (Adams & Cory 1998, Gross *et al.* 1999, Huang & Strasser 2000). Although the precise mechanisms are under debate, regulation of mitochondrial integrity and release of cytochrome c and other intermembrane proteins have been proposed the main functions of the Bcl-2-family. Bcl-2 family proteins are of particular interest in focal cerebral ischaemia since these proteins appear to act upstream of mitochondrial dysfunction and irreversible cell death.

THE PROTEINS: MEMBERS & CLASSIFICATION

Bcl-2, the founding member of the family was originally identified as the proto-oncogene involved in the t (14;18) translocation in human follicular β -cell lymphoma (Bakhshi *et al.* 1985). Bcl-2 has several

functions independent of its ability to suppress apoptotic cell death. These include: the regulation of calcium homeostasis (Lam *et al.* 1994, Martin *et al.* 1996), promotion of glutathione sequestration to the nucleus and modulation of antioxidant pathways (Hockenberry *et al.* 1993). A number of Bcl-2-related proteins have now been identified. Death inhibitors include Bcl-2 (Hockenberry *et al.* 1990), Bcl-X_L (Boise *et al.* 1993), Bcl-w (Gibson *et al.* 1996), Mcl-1 (Zhou *et al.* 1997), A1/Bfl-1 (Lin *et al.* 1993), adenovirus E1B 19K (Han *et al.* 1996), Epstein-Barr virus (EBV) BHRF1 (Henderson *et al.* 1993), *C. elegans* CED-9 (Hengartner & Horwitz 1994), Boo/DIVA (Inohara *et al.* 1998a) and Brag-1 (Das *et al.* 1996). Death promoters include Bax (Oltvai *et al.* 1993), Bak (Chittenden *et al.* 1995), Bcl-X_S (Minn *et al.* 1996), Bok/Mtd (Hsu *et al.* 1997a), Bad (Yang *et al.* 1995), Bik (Boyd *et al.* 1995), Bid (Wang *et al.* 1996), Bim (O'Conner *et al.* 1998), Hrk (Inohara *et al.* 1998b), Noxa (Oda *et al.* 2000), PUMA (Yu *et al.* 2001), BNip3 (Boyd *et al.* 1994), Nix (Matsushima *et al.* 1998, Imazu *et al.* 1998) and Bcl-Rambo (Kataoka *et al.* 2001). Splice variants of Bax (Apte *et al.* 1995), Bok (Hsu *et al.* 1997a), Nix, Bim (Liu *et al.* 2002) and more recently a neuron-specific splicing variant of Bak have been described (Sun *et al.* 2001). However, the functional implications of these splice variants is unclear. Bcl-2 related proteins share homology in one of four regions designated the Bcl-2 homology domains (BH1, BH2, BH3 and BH4; *Figure 1.4.1*). In the process of defining and classifying members of this superfamily on the basis of function and domain structure, two key findings were identified: (i) anti-apoptotic members harbour at least three BH domains – BH1, BH2 and BH3 – as well as a transmembrane domain, and (ii) the minimum requirement of pro-apoptotic members is a functional BH3 domain (Chittenden *et al.* 1995, Hunter & Parslow 1996, Zha *et al.* 1996a). Most members also harbour a C-terminal signal-anchor sequence that targets them predominantly to the outer mitochondrial membrane as well as the endoplasmic reticular membrane and nuclear envelope (Krajewski *et al.* 1993, Nguyen *et al.* 1993).

DIMERISATION: CONTROL OVER THE LIFE / DEATH RHEOSTAT

A distinguishing feature of the Bcl-2 family is the ability of its members to heterodimerise with other family members, and in some cases, to homodimerise with themselves. Several Bcl-2 family members were isolated by virtue of their ability to heterodimerise with Bcl-2 or Bcl-X_L in yeast two hybrid, co-immunoprecipitation, *in vitro* binding assays and cross-linking experiments (Oltvai *et al.* 1993, Minn *et al.* 1996). The concept of a life/death 'rheostat' was conceived by Oltvai and colleagues (1993) and expounded by Korsmeyer's group and others (Oltvai *et al.* 1994, Yang *et al.* 1996), where the sensitivity of a cell to a given death stimulus was proposed to be determined by the relative ratio of homo- and heterodimers. Mutagenesis studies have established that the BH1, BH2 and BH3 domains strongly influence protein-protein interactions (Chittenden *et al.* 1995, Yin *et al.* 1994). The BH1 and BH2 domains of Bcl-2-like molecules appear critical for both heterodimerization with pro-apoptotic members and cell survival (Sedlak *et al.* 1995, Yin *et al.* 1994). Although multidomain members, such

as Bax, contain BH1 and BH2 domains, the BH3 domain appears critical for induction of cell death and heterodimerization with anti-apoptotic members (Chittenden *et al.* 1995, Zha *et al.* 1996). One exception applies to the BH3-only member, Bid, which appears unique in that it can bind both anti-apoptotic and multidomain members (Wang *et al.* 1996). Whether other BH3-only members have the ability to bind both anti-apoptotic and pro-apoptotic multidomain members is unclear, as are the functional implications of Bax:BH3-only protein interactions.

LEVELS OF REGULATION

The Bcl-2 protein family are of particular interest in stroke research because they appear to be regulated by post-translational modifications, rather than protein synthesis *per se*. In addition, all pro-apoptotic members reported appear to share a common function – increased localisation at the outer mitochondrial membrane following an apoptotic stimulus. The fact that these proteins do not require *de novo* synthesis and act upstream of mitochondrial dysfunction and irreversible cell death, suggests that targeting this family of regulatory proteins or molecules regulating their activity might prove beneficial in stroke research.

TRANSCRIPTIONAL REGULATION

Numerous studies indicate that Bcl-2-like family proteins can be regulated at the level of transcription (Ohta *et al.* 1995). The tumour suppressor, p53, has been reported to mediate transcriptional activation of the human Bax promoter resulting in upregulation of Bax protein expression (Miyashita & Reed 1995). Transcriptional activation of BNip3 and Nix were also recently reported (Bruick 2000, Yu *et al.* 2001). BH3-only members, Noxa (Oda *et al.* 2000) and PUMA (Nakano & Vousden 2001, Yu *et al.* 2001), were also reported to be regulated by p53.

POST-TRANSLATIONAL MODIFICATIONS

Strack *et al.* (1996) first demonstrated that Bcl-2 cleavage promotes cell death. The cleavage site was subsequently reported in the variable loop region which resulted in exposure of the BH3 domain, thus converting Bcl-2 into a death promoting molecule (Cheng *et al.* 1997, Fujita & Tsuruo 1998). *C. elegans* homologue of Bcl-2, CED-9, is cleaved by CED-3 (Xue & Horvitz 1997). Caspase-8-mediated cleavage of Bid has also been reported during death receptor activation (Li 1998, Schmitz 1999). Bax can also be cleaved by caspases and by the calcium-activated protease calpain (Wood *et al.* 1998) to produce an 18kDa fragment of Bax, with increased cytotoxicity (Wood & Newcomb 2000).

Protein phosphorylation is the most common cellular mechanism for post-synthetic regulation of protein function. Several Bcl-2 family proteins appear to be regulated by phosphorylation. For example, phosphorylation abrogated the anti-apoptotic function of Bcl-2 and Bcl-X_L (Haldar *et al.* 1996, Chen *et al.* 1996). In contrast, phosphorylation was reported to abrogate the pro-apoptotic activity of BH3-only proteins, Bad (Wan *et al.* 2002) and Bik (Verma *et al.* 2000). Whereas, following an apoptotic stimulus, Bad is dephosphorylated and free to promote cell death (Wang *et al.* 1996, Harada *et al.* 1999). However, phosphorylation of another BH3-only member, Bid, rendered this molecule resistant to cleavage-mediated activation (Desagher *et al.* 2001). Therefore, a protein-specific response to cleavage and phosphorylation status appears to exist between the various Bcl-2 members.

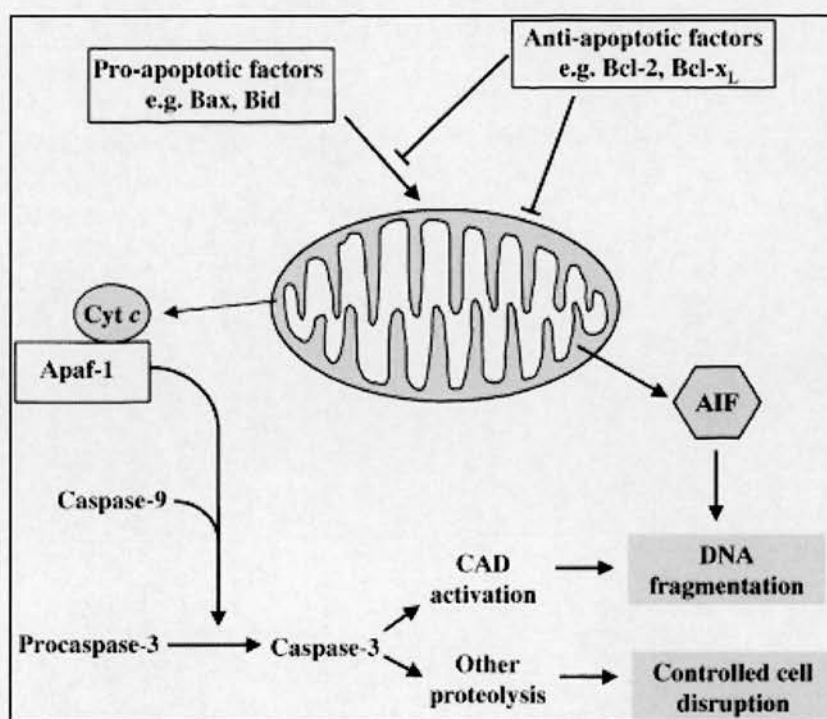


Figure 1.4.2: Bcl-2 family protein regulation – upstream of irreversible cell death.

The Bcl-2 family regulate mitochondrial integrity. Following an apoptotic stimulus, pro-apoptotic members accumulate at the mitochondria and promote cytochrome c release. In the presence of ATP, apoptosome formation catalyses caspase-9 activation, which, in turn, activates downstream effector caspases, such as caspase 3.

Multidomain proteins, such as Bax and Bak, are inactive in healthy cells. In normal tissues Bax is predominantly cytosolic and a monomer (Hsu & Youle 1998, Antonsson *et al.* 2000). Activation and translocation of Bax is accompanied by conformational changes in the N- and C-terminal of this protein before insertion into the outer mitochondrial membrane (Nechustan *et al.* 1999). Apoptosis has been associated with conformational changes of Bax and Bak (Griffiths *et al.* 1999, Suzuki *et al.* 2000, Antonsson *et al.* 2001). In addition, non-ionic detergents, such as TX-100 and NP-40, can induce exposure of the N-terminal of Bax, as evidenced with the use of the conformational-specific

monoclonal antibody 6A7 (Hsu & Youle 1997) and therefore, should be excluded from extraction buffers when investigating Bax activation. Therefore, all cells were homogenised in a detergent-free extraction buffer throughout this thesis. Intracellular redistribution during stress conditions is a common event for many regulators of the apoptotic cell death cascade. The anti-apoptotic effect of Bcl-2 is considered largely dependent on its membrane localisation (Nguyen *et al.* 1994, Tanaka *et al.* 1993). Also, Bax expression *per se* is not lethal to cells; however Bax translocation towards the mitochondria during apoptosis has been reported to be an important event for Bax-mediated cell death (Wolter *et al.* 1997, Goping *et al.* 1998, Gross *et al.* 1998, Khaled *et al.* 1999). Deletion of the carboxy-terminal hydrophobic region of Bax was observed to prevent Bax redistribution and to abrogate its pro-apoptotic activity, thus underscoring the importance of organelle binding for the activity of this molecule. While Bcl-2 and Bcl-X_L have been reported localised to the outer mitochondrial membranes of resting cells, many pro-apoptotic members (e.g. Bax, Bad and Bcl-X_S) appear cytosolic and only targeted to the mitochondria upon receipt of an apoptotic stimulus (Jia *et al.* 1999). The aim of this thesis was to investigate the role of the Bcl-2 family in focal cerebral ischaemia and to characterise the mechanism of action of several pro-apoptotic Bcl-2 family proteins. While protein-specific responses have been reported to a given apoptotic stimulus, mitochondrial accumulation appears to be a common event in the signal transduction of these proteins pro-apoptotic response (Figure 1.4.2). As such, translocation towards the mitochondria was investigated as a more sensitive measure of Bcl-2 family protein activation in this thesis.

BCL-2 CONTROL OVER THE MITOCHONDRIA

The point of no return that commits the cell to die may well be translocation of cytochrome c into the cytosol (Oltvai *et al.* 1993), where it is then free to promote apoptosis via the mitochondrial pathway. The pro-apoptotic Bcl-2 family proteins execute their function at the level of the mitochondria, although the exact mechanism is not understood. Mitochondrial permeability transition, alterations in subcellular distribution of calcium and hydrogen ions, and the subsequent $\Delta\Psi_m$ collapse have been associated with apoptosis (Marchetti *et al.* 1996, Zamzami *et al.* 1996), and Bcl-2 or Bcl-X_L over-expression, for the most part, prevents these events from occurring (Kluck *et al.* 1997, Yang *et al.* 1997). Bax translocation with concomitant cytochrome c release has been reported a pivotal event in mitochondrial-mediated cell death (Rosse *et al.* 1998). Anti-apoptotic members inhibit cytochrome c release, although the precise mechanism remains controversial (Vander Heiden *et al.* 1997). Since several pro- and anti-apoptotic members have been reported to form pores in synthetic membranes, it was hypothesised that these molecules might regulate mitochondrial integrity by several mechanisms. Three possible mechanisms include (i) binding within the hydrophobic pocket of the anti-apoptotic protein, creating a non-functional or dysfunctional pore, possibly with altered ion selectivity, thus compromising mitochondrial integrity, (ii) heterodimerization-induced conformational changes in the

anti-apoptotic protein, thus rendering it incapable of mitochondrial insertion or (iii) the formation of a multidomain Bax/Bak-like pore. Many pro-apoptotic family members lack the channel-forming BH1 and BH2 domains in their entirety. These BH3 domain-only proteins can heterodimerise with anti-apoptotic Bcl-2 family members, but are unable to homodimerise. It was shown that proteins such as Bim and Bad translocate to the mitochondria following an apoptotic stimulus, whereby they bind to anti-apoptotic Bcl-2 or Bcl-X_L (Wang *et al.* 1996). However, in addition to heterodimerising with Bcl-2 and Bax, BH3-only member, Bid, was reported to directly insert in isolated mitochondria, and promote cytochrome c release (Luo *et al.* 1998). Therefore, the concept of a simple 'rheostat' to explain the Bcl-2 family regulation of cell integrity might not hold true. Thus, the fabled point-of-no-return in cell death should perhaps be equated with reaching a certain threshold of activation of proteases and other effector molecules, and the role of the Bcl-2 family members may be to regulate this 'apoptotic threshold', possibly at the level of the mitochondrion. Pro-apoptotic Bax, Bad and Nix were chosen to represent the multi-domain, BH3 domain (without TM domain) and BH3 domain only (with TM domain), respectively (see Figure 1.4.1). At the time of study, evidence to support the hypothesised *in vitro* and *in vivo* molecular circuitry of these proteins was limited, with most published data being restricted to Bax, which appeared both complicated and contradictory. Importantly, most *in vitro* data employed artificial conditions, such as over-expression or mutational studies, with measurements from single cell, isolated organelles or artificial lipid membranes, raising questions regarding the physiological relevance of such data. Nevertheless, early *in vitro* studies reported Bax translocation from the cytosol to mitochondria to be a common event following several *in vitro* apoptotic stimuli (Wolter *et al.* 1997, McGinnis *et al.* 1999). Over-expression of most pro-apoptotic Bcl-2 family members investigated to date have reported mitochondrial accumulation to be a fundamental event in their pro-apoptotic cascade. However, at the time of study no such evidence had been provided for such a mechanism to exist within ischaemic stroke tissue. Therefore, it was necessary to characterise the *in vitro* and *in vivo* mechanism(s) of action of Bax, Bad and Nix, as examples of each subgroup, where cell / stimulus specific responses might exist.

1.5 ISCHAEMIC DAMAGE - NECROSIS OR APOPTOSIS?

Damage in the penumbra, but less commonly in the core lesion, can be ameliorated by a range of pharmacological or physical interventions initiated around the time of vessel occlusion (Dirnagl *et al.* 1990, Gill *et al.* 1992, Park & Hall 1994). Thus, although the consequences of permanent vessel occlusion in the core and penumbra appear similar, differences in the underlying mechanism of cell death likely exist. Neuronal death following an ischaemic insult can either be immediate, as observed in the core lesion, or delayed, as reported in the penumbra. The core lesion has been reported to contain primarily necrotic-like cells, whereas the penumbra is generally considered to contain electrically silent neurones but with their ionic gradients largely intact, which may ultimately die by

either necrosis or apoptosis (Beilharz *et al.* 1995, Kihara *et al.* 1994, Onteniente *et al.* 2003). Over time, the infarct grows in size where cells are recruited into the core lesion. This recruitment process constitutes a critical period in which lesion expansion might be inhibited if reperfusion is initiated within 2-3 h onset of ischaemia (Gingsberg & Pulsinelli 1994). That a penumbral zone exists and is amenable to therapeutic intervention is substantiated by the number of experimental approaches using animal models of stroke. A 50% or greater reduction in infarct volume has been reported following mild hypothermia, Ca^{2+} channel blockers, and oxygen free radical scavenger therapy, which appears to coincide with the measured penumbral volume (Gingsberg & Pulsinelli 1994). Evidence suggests that neurones continue to die in the human stroke patient for up to 10 days after infarction (Saunders *et al.* 1995). The single most important factor in promoting lesion expansion was reported to be the generation of peri-infarct depolarisations, which produces a spreading depression further aggravating the cellular energy crisis in the ischaemic tissue (Back *et al.* 1994, Hossmann *et al.* 1994, Hossmann *et al.* 1996). Other putative mechanisms include inflammation (Becker *et al.* 1998) or the combination of excitotoxicity with programmed cell death (Choi *et al.* 1996, Leist & Nicotera 1998, Lipton *et al.* 1999). The release of excitatory amino acids from the ischaemic core is thought to evoke intracellular calcium flooding which, in turn, promotes cytochrome c release from the mitochondria and apoptotic cell death (Krajewski *et al.* 1999). However, the exact contribution of these events to the overall ischaemic damage is unknown.

Terminal-deoxynucleotidyl-transferase-mediated-dUTP nick end labelling (TUNEL) staining, which detects single and double DNA strand breaks, is a common marker of apoptosis and is best supported by electron microscopy (Banasiak *et al.* 2000). Apoptotic cells defined this way have been found scattered throughout the vascular territory following permanent (Asahi *et al.* 1997, Linnik *et al.* 1993, Murakami *et al.* 1998) and transient MCA occlusion in rodents (Charriaut-Marlangue *et al.* 1995, Charriaut-Marlangue *et al.* 1996, Li *et al.* 1995, MacManus *et al.* 1997). The therapeutic challenge of targeting delayed neuronal cell death, whether necrotic or apoptotic is the therapeutic challenge in stroke research. Necrosis is the predominant mechanism that follows acute, permanent vascular occlusion. However, an increasing body of data suggests that apoptotic cell death mechanisms might be 'unmasked' during less severe conditions of focal cerebral ischaemia (Akins *et al.* 1996, Charriaut-Marlangue *et al.* 1996, Linnik & Ringer *et al.* 1999). Key components of the apoptotic cell death program have now been detected in animal stroke models (Du 1996, van Lookeren & Gill 1996, MacManus *et al.* 1997, Nicotera 1999, Banasaik *et al.* 2000, Rabuffetti *et al.* 2000). For example, neuronal protection was offered even when caspase-3 inhibitors were added up to 9 h after onset of transient (30 min) ischaemia in mice (Fink *et al.* 1998). Increased levels of free radicals, extracellular glutamate, pro-inflammatory cytokines, or lipid peroxidation products generated within the ischaemic core lesion might mediate apoptotic cell death in the penumbra (Nicotera *et al.* 2000). In addition to the collapse of chromatin into highly condensed electron-dense masses, the appearance of DNA 'ladder' fragments resulting from activation of a calcium-dependent endonuclease

was initially considered the primary biochemical event occurring in apoptosis (Arends *et al.* 1990). Oligonucleosomal DNA degradation has been reported in the ipsilateral hemisphere after 24 h - 48 h ischaemia (Linnik *et al.* 1993, Tominaga *et al.* 1993, MacManus *et al.* 1997, Charriaut-Marlangue *et al.* 1995). Activation of several apoptosis-regulatory gene products have been reported within these time points (Lipton *et al.* 1999, Schulz *et al.* 1999, Sharp *et al.* 2000). These include: immediate early genes, *c-jun*, *c-fos* and *junB*, (Gillardon *et al.* 1996) heat shock proteins (Bates 2001, Zee *et al.* 2002), and pro-apoptotic Bcl-2 family members (MacManus *et al.* 1997, Gillardon *et al.* 1996). Increased TUNEL positivity has been reported localised to the inner boundary zones of the infarct. Based on these findings, Li *et al.* (1995) proposed that apoptosis accompanies necrosis following focal cerebral ischaemia.

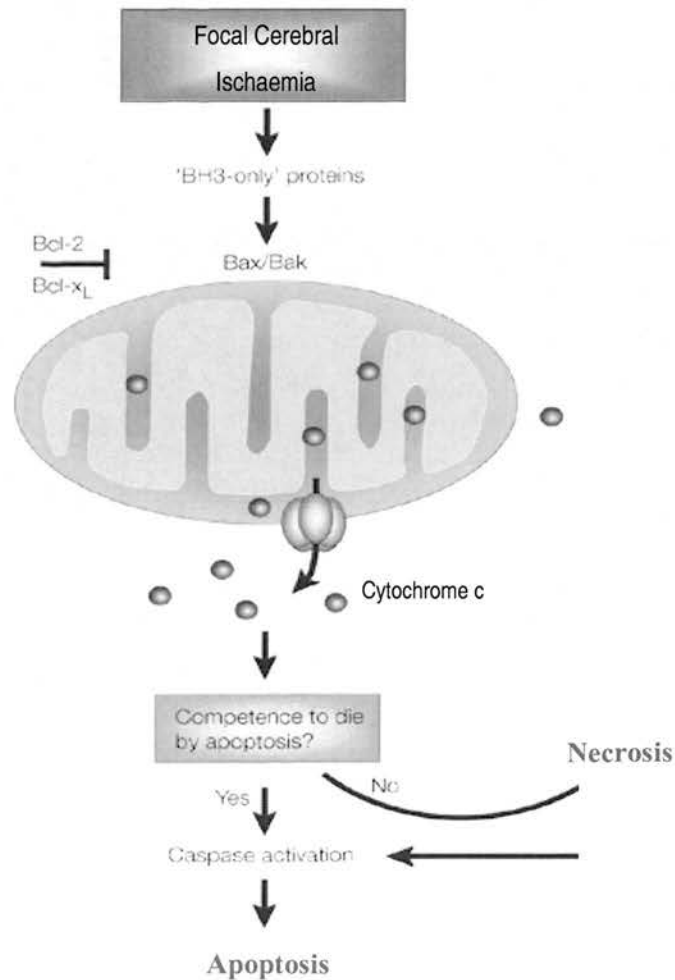


Figure 1.5.1: Bcl-2 family regulation of ischaemic cell death.

The pro-apoptotic Bcl-2 family might regulate both apoptotic and necrotic cell death mechanisms during focal cerebral ischaemia, depending on residual energy levels within the ischaemic tissue.

A key study reported a progressive and delayed cell death response up to 14 days after an initial occlusion period of 30 min in rats (Du *et al.* 1996). At this time point, the lesion volume was comparable with a mature lesion observed following permanent ischaemia, and encompassed the entire MCA territory. This delayed cell death was attributed to the activation of apoptotic-like cell death mechanisms. However, this study has proved difficult to reproduce (Henshall, 1997, McCarter, 2001). Among all the apoptosis-based drug therapies, strategies that target caspases are at the forefront for anti-apoptosis therapies in numerous diseases. Caspase inhibitors markedly reduce infarct volume and decrease neurological deficits (Hara *et al.* 1997), which further supports a role for apoptosis in focal ischaemic cell death (Endres *et al.* 1998, Rabuffetti *et al.* 2000, Weissner *et al.* 2000). At least in some cases however, caspase inhibition only delays cell death and cells eventually die with morphologically different features (Hirsch *et al.* 1997, Leist *et al.* 1997). For example, under conditions of ATP depletion, as occurs during ischaemia, cells initially triggered to undergo apoptosis might be forced to die by a more passive necrotic-like cell death (Leist *et al.* 1997, Figure 1.5.1).

Indeed, whether sufficient ATP levels exist within the ischaemic brain to enable activation of such an energy-consuming mechanism underlies one of the major arguments against a role for apoptosis in focal cerebral ischaemia. It is generally accepted that permanent vessel occlusion results in a rapid decline in ATP levels in the core, with less severe reductions in the penumbra (Folbergrova *et al.* 1992, Nakai *et al.* 1995). In contrast, reperfusion in at least some studies was associated with almost complete restoration of ATP levels in the penumbra, with at least partial recovery in the core lesion (Selman *et al.* 1990, Hata *et al.* 2000b). Therefore, it appears possible that residual energy, at least in less compromised tissue, might permit execution of apoptotic cell death. However, irrespective of the final execution pathway (mitochondrial-dependent/independent) the Bcl-2 family of regulatory proteins might play a critical role in stroke pathology, upstream of mitochondrial dysfunction and irreversible cell death. Several key findings support an essential role for the Bcl-2 family in stroke. These include: reduced infarct volumes in Bcl-2 over-expressing mice (Martinou *et al.* 1994), early Bax upregulation and concomitant cytochrome c release (Gillardon *et al.* 1996, Fugimura *et al.* 1998, Prakasa *et al.* 2000) and reduced infarct volumes in Bax null mice (Linnik & Ringer 1999).

THESIS OVERVIEW

This thesis investigated the role of the pro-apoptotic Bcl-2 family proteins, Bax, Bad and Nix, in focal cerebral ischaemia. In *Chapter 2*, reproducible rat stroke models, using the intraluminal monofilament MCA occlusion model were established. Preliminary biochemical investigations were performed to investigate whether a pro-apoptotic response could be detected in the vascular territory (cortex and

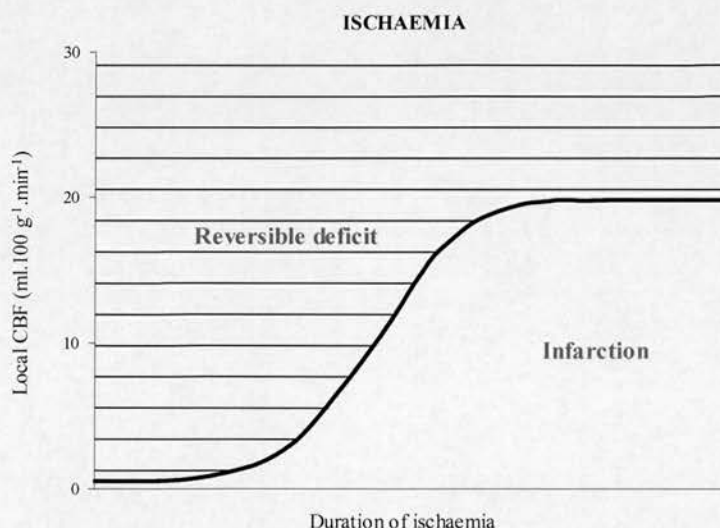
striatum) of the occluded vessel. Using changes in total protein expression of Bax, Bad and Nix as well as cleaved caspase-3 as a measure of Bcl-2 family activity and apoptotic cell death, respectively, no evidence was provided to support a significant role for apoptosis in the ischaemic stroke model investigated. Several *in vitro* models of apoptosis were subsequently set-up. Protocols for subcellular fractionation and confocal microscopy were established as a more accurate measure of Bcl-2 family protein activity. As such, the *in vitro* mechanisms of action for Bax (Chapter 3), Bad (Chapter 4) and Nix (Chapter 5), were characterised as examples of *multidomain*, *BH3-only* (without TM domain) and *BH3-only* (with TM domain) family members.

Several models of focal cerebral ischaemia were established, using varied occlusion and reperfusion durations in attempts to optimize detection and characterisation of the putative role of pro-apoptotic Bcl-2 family proteins in focal cerebral ischaemia. Since *in vitro* studies supported a pivotal role for mitochondrial accumulation following an apoptotic stimulus, a subcellular fractionation protocol was optimized in rat brain tissue. These studies identified similar *in vitro* and *in vivo* mechanisms of action for each protein investigated, thus supporting the use of these *in vitro* models of apoptosis for further characterisation of the molecular circuitry of Bax, Bad and Nix. Importantly, a progressive pro-apoptotic Bcl-2 family response for each protein was observed with increased ischaemic duration. This was characterised by upregulation and subsequent mitochondrial accumulation. More importantly, this protein response correlated with increased cleaved caspase-3 immunoreactivity, apoptotic body formation and lesion expansion. Therefore, these findings provide strong evidence to support a role for the pro-apoptotic Bcl-2 family in the pathophysiology of focal cerebral ischaemia. Finally, and perhaps most intriguing, was the finding that increased reperfusion duration in these models was associated with activation of an intrinsic survival mechanism. This was mediated by a novel protein kinase B / Akt signalling mechanism, which appeared to be, at least partially, regulated by inhibition of the pro-apoptotic Bad response. Since *in vitro* and *in vivo* studies demonstrated that this event likely occurs upstream of mitochondrial dysfunction and irreversible cell death, this novel regulatory mechanism might represent a potential therapeutic target in ischaemic stroke research.

CHAPTER 2: FOCAL ISCHAEMIC STROKE

INTRODUCTION

Occlusion of a major vessel leads to tissue infarction that typically involves a severely damaged core (focus) with a surrounding 'perifocal' region exposed to less profound reductions in cerebral blood flow (Jacewicz *et al.* 1992, Memezawa *et al.* 1992). Middle cerebral artery (MCA) occlusion, the most common model of experimental stroke, commonly produces a cortical and striatal lesion, encompassing the vascular territory of the MCA (Tamura *et al.* 1981). The lesion expands as the period of occlusion is extended up to several hours, at which point no further expansion occurs. However, substantial differences have been reported in their underlying pathophysiology. In humans, reversal of the occlusion can occur either spontaneously, or following treatment with thrombolytic agents. Clinical (ECASS Study Group 1995, NINDS rt-PA Stroke Study Group 1995, Hacke *et al.* 1999) and experimental (Chopp *et al.* 1999) studies have shown that early restoration of blood flow can salvage perifocal ischaemic brain tissue. However, reperfusion if delayed has been reported among other things, to promote blood-brain barrier (BBB) disruption, haemorrhagic transformation and oedema formation (Hacke *et al.* 1999). Ischaemic duration and reperfusion likely influence ischaemic tissue damage. The temporal profile of ischaemic damage appears to vary between species, where damage has been reported to occur more rapidly in rodents compared to primates (*Figure 2.1*).



*Figure 2.1: Relationship between ischaemic duration and severity of insult (Halestrap *et al.* 2000).*

Ischaemic damage in the rat appears dependent on residual levels of cerebral blood flow (CBF) in the occluded artery and ischaemic duration. (Figure by Halestrap *et al.* 2000, based on a descriptive account of lesion damage after MCA occlusion in non-human primates by Garcia & Kamijyo 1974).

Although considerable progress has been made in identifying processes that contribute to ischaemic tissue damage, these mechanisms remain incompletely defined. Animal models of focal cerebral ischaemia have facilitated characterisation of the ischaemic cell death cascade. Method of assessment, the strain of species used, operator and other factors may influence infarct volume and the observed evolution of infarction (Brint *et al.* 1988). Classical studies by Sundt *et al.* (1969, 1972) reported MCA occlusion periods of 1 h and 6 h in cats and non-human primates, respectively, which did not lead to infarction. Occlusion studies in non-human primates often led to nonfatal hemiparesis, which is proposed to more closely reflect human stroke. Early MCA occlusion studies in these subjects determined 4-8 h ischaemia as the minimum duration required to yield the maximum extent of infarction (Crowell *et al.* 1970, 1981). In contrast to poorly defined lesions observed within the first 12 h of MCA occlusion in rats (Bederson *et al.* 1986), a well-demarcated lesion is commonly observed after 24 h ischaemia (Garcia and Kamijyo 1974, Garcia *et al.* 1994, Zhang *et al.* 1994, Hoehn-Berlage *et al.* 1995). Further lesion expansion has been reported in some stroke studies up to 72 h ischaemia, but attributed to oedema formation (Chen *et al.* 1993, Zhang *et al.* 1994). Kirino (1988) specifically investigated slow neuronal damage at intervals up to 6 months after permanent MCA occlusion in rats, and concluded that the lesion volume was maximal by 24 h ischaemia. As such, 24 h ischaemia is a commonly used stroke model used to characterise the pathophysiology of ischaemia stroke (Tamura *et al.* 1981, Kaplan *et al.* 1991).

Neuronal cell death that follows focal cerebral ischaemia has been commonly described as necrosis rather than apoptosis. However, based on emerging evidence an active type of cell death, reminiscent of apoptosis, might account for at least some of the tissue damage observed in the core ischaemic lesion (1:9 incidence) and surrounding penumbra (1:6 incidence). The Bcl-2 protein family has been implicated in the pathophysiology of stroke (Linnik *et al.* 1993, Gillardon *et al.* 1996 and Raghupathi *et al.* 2000), where over-expression of anti-apoptotic Bcl-2 and Bcl-X_L in rodents results in smaller infarct volumes (Martinou *et al.* 1994, Linnik *et al.* 1995, Lawrence *et al.* 1996, Weissner *et al.* 1999). More recently, over-expression of the cell death suppressor Bcl-w was reported to offer protection after transient MCA occlusion in mice (Yan *et al.* 2000). DNA fragmentation and increased levels of pro-apoptotic Bax mRNA expression have been reported as early as 6 h after permanent MCA occlusion in the rat (Gillardon *et al.* 1996). These findings support a role for the Bcl-2 family in the pathophysiology of focal cerebral ischaemia. Therefore, based on available published histological and biochemical data, permanent and transient rat stroke models were set-up and putative pro-apoptotic Bcl-2 family responses were investigated following 24 h ischaemia.

AIMS

The aims of this chapter were to (a) obtain reproducible histological data of permanent and transient MCA occlusion in a rat stroke model which was comparable to other reported data, (b) investigate the evolution of damage in permanent and transient stroke models and (c) based on the above findings, identify a model of focal cerebral ischaemia with which to further investigate the putative pro-apoptotic Bcl-2 family response(s) to focal cerebral ischaemia.

MATERIALS & METHODS

Permanent (PMF) and transient (TMF, 2 h) monofilament occlusion of the right MCA was induced in male Sprague Dawley rats (275-315g, aged 8-11 weeks) under 5% halothane and maintained under 2% halothane (70% N₂O : 30% O₂) anaesthesia.

Rat Permanent Monofilament Occlusion

Focal cerebral ischaemia was induced using the method described by Zea Longa *et al.* (1989) with modifications. A midline incision and division of the omohyoid muscle exposed the left common carotid artery (CCA). The external carotid artery (ECA) was identified, dissected free from surrounding connective tissue and ligated distally with 6/0 silk suture (Ethicon, UK). A second 6/0 silk suture was tied loosely at the bifurcation of the ECA and the internal carotid artery (ICA). A 4/0 silk suture (Ethicon) was placed around the ICA and tension applied to it to control bleeding. An angled atraumatic microvascular clip (6 mm micro-serrefine, Fine Scientific Tools) was placed across the CCA and a small incision made in the ECA at approximately the level of the origin of the occipital artery. A nylon monofilament (Ethicon), knotted at 18-19 mm, coated with poly-L-lysine (Sigma) and tip rounded near flame was introduced into the lumen of the ICA via the ECA. The suture was carefully advanced into vasculature of the brain to a point where it occluded origin of the middle cerebral artery (MCA) (*Figures 2.2, 2.3 and 2.4*). The silk suture at the bifurcation of the ECA and ICA was tightened around the monofilament. The neck wound was sutured with 4/0 silk sutures (Ethicon) and the animal allowed to recover in an incubator where normothermia (23°C) was maintained. The common carotid artery of sham-operated animals was exposed, the monofilament transiently inserted, retracted back towards the external carotid artery and tied off. A rectal probe was used to maintain the animal's body temperature at 37°C ± 0.5°C throughout both surgical procedures. Re-introduction of blood supply through the common carotid artery was visualized under a light microscope.

Rat Transient Monofilament Occlusion

Surgery was performed as described for the permanent monofilament model. Two hours (h) post occlusion, the animal was re-anaesthetized, neck wound re-opened and the monofilament retracted to the point where the tip remained in the ECA and then cut. Blood flow in the ICA was visibly re-established in each case. Following surgery, the neck wound was sutured and the animal placed in an incubator to recover. The effect of various occlusion times on the volume of damage in the transient model was examined. The monofilament was withdrawn after a given occlusion duration and the brains processed as described below. Evans Blue (2 %, Sigma) was injected i.v. into anaesthetized animals 10 min prior to perfusion fixation, and used to determine the placement of the monofilament in the vasculature of transient MCA occlusion animals.

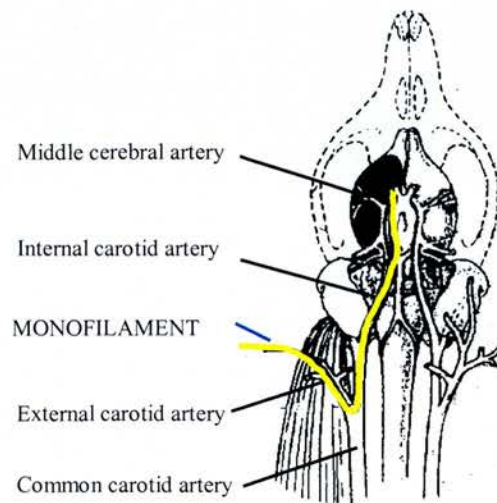


Figure 2.2: Placement of intraluminal monofilament for permanent and transient middle cerebral artery occlusion (Diagram modified from Zea Longa et al. 1989).

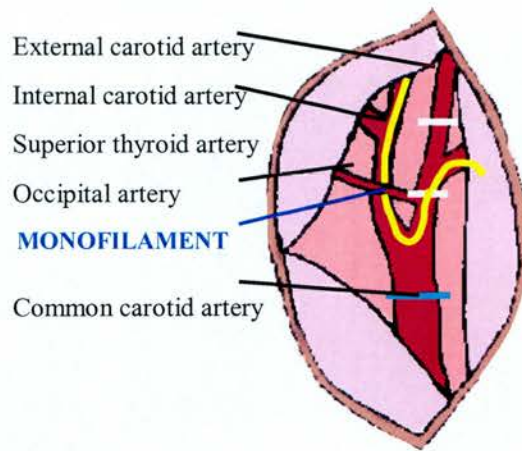


Figure 2.3: Surgical exposure of the arteries in the neck of the rat.

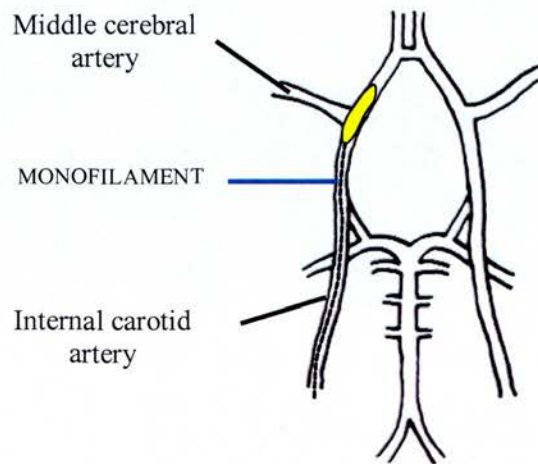


Figure 2.4: Placement of the monofilament in the vasculature of the rat brain such that it occludes the origin of the middle cerebral artery.

Histological Assessment of Damage

Animals were deeply anaesthetized by intraperitoneal injection (*i.p.*) of sodium pentobarbitone (60 mg.kg⁻¹, Rhône Mérieux). The thoracic cavity of the animal was opened, a needle connected to a pump (Watson Marlow 502S) inserted through the apex of the left ventricle into the ascending aorta and a small incision was made in the right atrium. Animals were perfused initially with 20 ml heparinised (10 IU.ml⁻¹) phosphate buffered saline (PBS) followed by 200 ml of 4 % paraformaldehyde (PFA) in PBS. Following perfusion, brains were removed, placed in 20 % sucrose

in PFA/PBS solution and stored at 4°C for at least 24 hours. Brains were removed from sucrose/PFA/PBS solution, frozen at -42°C in isopentane and mounted in plastic embedding matrix (M1 embedding Matrix, Lipshaw) on a chuck. 20 µm cryostat sections were taken at -20°C and placed on gelatine coated slides (5 %) and air dried at 20-30°C. Slides were subsequently stained with thioinin (*see Appendix A*).

Animals were allowed to recover for 24 h or 72 h after the onset of ischaemia. Infarct margins were identified by thionin staining and brain damage quantified using a microcomputer imaging device (MCID; *see Appendix B*). Volume of damage (mm³) was expressed as the mean ± standard error of the mean (s.e.m.). Statistical analysis was performed using analysis of variance (ANOVA) with *post hoc* Student-Newman-Keuls test (SigmaPlot version 8, Sigma Stat version 3.0).

Biochemical Assessment of Damage

The cortex and striatum of sham-operated and ischaemic rat brains (2h MCA occlusion / 22 h reperfusion) were isolated and transferred to a glass Dounce homogeniser (1mL, Jencons) containing an ice-cold extraction buffer (10mM Tris-Cl (pH 7.4), 125mM Na⁺Cl⁻, 1mM EGTA, 1mM EDTA, 0.1mM DTT, 20µM lactacystin, 1mM PMSF, phosphatase inhibitors (2.5mM sodium pyrophosphate, 1mM β-glycerolphosphate and 1mM sodium orthovanadate) and 10µg/mL each protease inhibitors – aprotinin, pepstatin and leupeptin). Cells were lysed by 6 ‘up/down’ strokes using a loose-fitting pestle and 10 ‘up/down’ strokes using a tight-fitting pestle. Whole cell lysates were subsequently passed through 0.35mm gauze to remove unbroken cells, blood cells and general cell debris. Whole cell lysates were spun at 13, 000 rpm for 5 minutes using a benchtop centrifuge (Biofuge *fresco*, Heraeus Instruments) to remove insoluble material. Protein content was then measured using a standard colorimetric BCA assay (Pierce) and analysed using Western blotting (30µg/lane) for changes in protein expression levels of pro-apoptotic Bax, Bad and Nix (*see Appendix C*). After blocking, membranes were incubated overnight at 4°C with one of the following rabbit polyclonal primary antibodies: anti-cleaved caspase-3 (1:1000; New England BioLabs), anti-Bax NT (1:500; Upstate Laboratories), anti-Bad (1:000; Cell Signalling Technology) or anti-BNip3L (1:1000; ψProSci Inc.). Membranes were also incubated with mouse primary antibody anti-β-tubulin (1:1000; Sigma Aldrich) to ensure equal loading and protein transfer. The immunoreactive proteins were visualized using the appropriate horseradish peroxidase-coupled anti-rabbit or anti-mouse Ig secondary antibody (1:2500; Amersham Life Sciences) and enhanced chemiluminescence (ECL, Amersham Life Sciences).

RESULTS

VOLUME & DISTRIBUTION OF DAMAGE

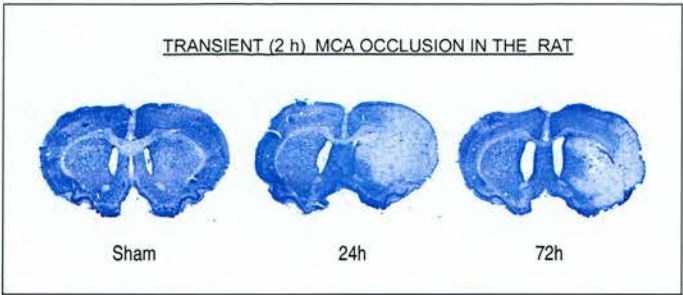


Figure 2.5: Distribution of damage in a MCA occlusion model of transient ischaemic stroke.

Thionin staining (20µm coronal sections) identified a lesion (area of pallor) encompassed the cortex and striatum. The lesions observed after 24 h and 72 h transient (2 h) ischaemia appeared similar in overall pattern and distribution of damage (when all 9 stereotaxic brain regions were assessed). Oedema was observed in both ischaemic rat brains, as evidenced by the reduced ventricular volumes in each brain. Each brain section was taken from three representative brains.

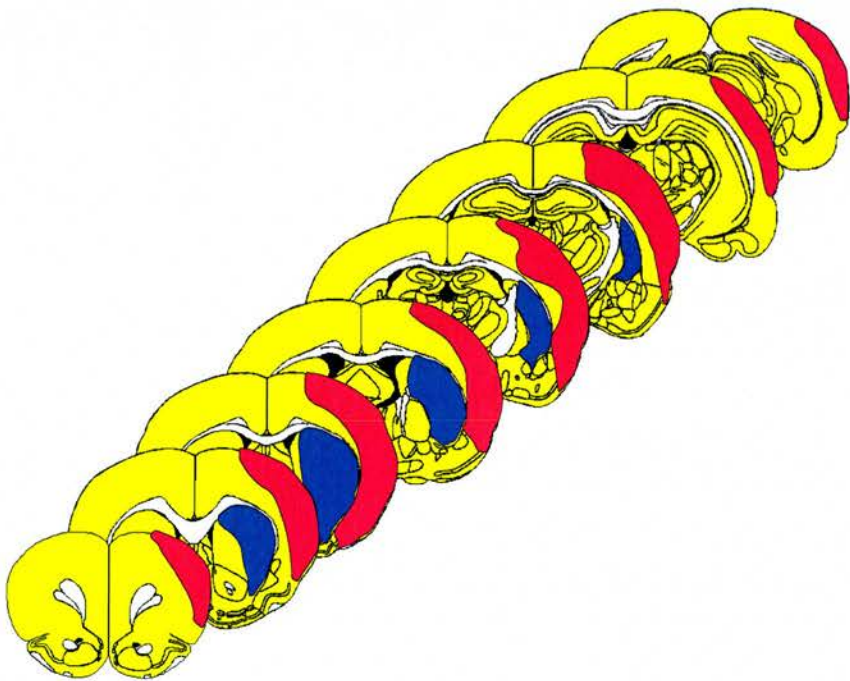


Figure 2.6: Schematic representation of the MCA territory in the rat

Cortical (red) and striatal (blue) tissue encompassing the entire middle cerebral artery (MCA) territory in the rat that is at risk during MCA occlusion (Sharkey & Butcher 1995).

Initial experiments conducted were aimed at establishing a reliable protocol for the intraluminal monofilament model. Once the permanent monofilament (PMF) model was established, the transient monofilament (TMF) model was optimized and ischaemic damage was compared between the two stroke models. Brain damage was readily discernable in thionin stained 20 μ m coronal sections from both PMF and TMF models 24 h and 72 h post-occlusion by light microscopy (*Figure 2.5*). In each model, the lesion was restricted to the vascular territory of the MCA. In all models, the lesion affected the lateral parts of the frontal cortex extending through the parietal into insular cortex rostrally and caudally into the occipital cortex of the ipsilateral hemisphere. There was also evidence of infarction within the dorsolateral caudate nucleus (striatum). No damage was detected in the contralateral hemisphere. The observed temporal and spatial profile of damage was typical of an MCA occlusion, encompassing the entire vascular territory (*Figure 2.6*).

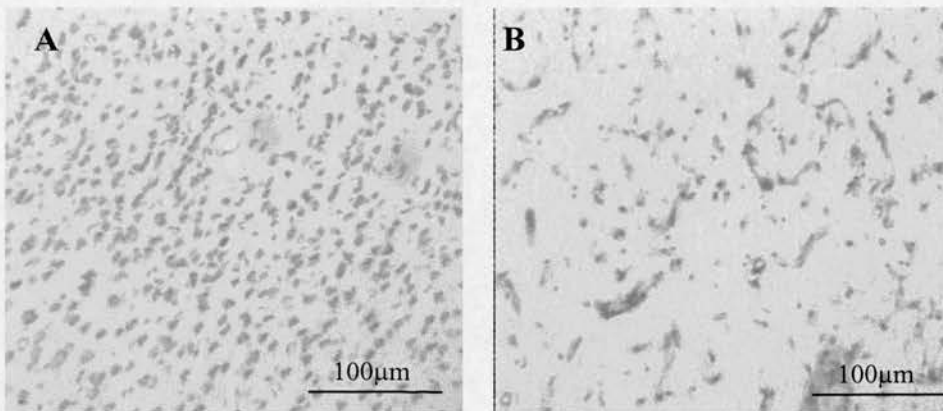


Figure 2.7: Morphological assessment of cortex 24h after onset of transient (2 h) MCA occlusion
Thionin stained cryostat section (20 μ m) showing the non-ischaemic contralateral hemisphere (A) and ischaemic damage in ischaemic ipsilateral hemisphere (B) 24 h following transient (2 h) monofilament occlusion of the middle cerebral artery.

The distribution of damage observed in thionin-stained brain sections 24 h and 72 h after onset of transient (2 h) ischaemia was investigated. Although extent of infarction varied slightly between models, the boundaries of the infarct were clearly identified 24 h after ischaemic onset confirming that any damage at this time point is representative of stroke-mediated events (*Figure 2.5*). ‘Ghost-like’ cells and general cell debris, indicative of cell lysis were detected within the infarcted area of each stroke model investigated, and displayed characteristics of a necrotic-like morphology. Pyknotic cells were not detected at time points investigated (*Figure 2.7*). There was no evidence of damage in sham animals.

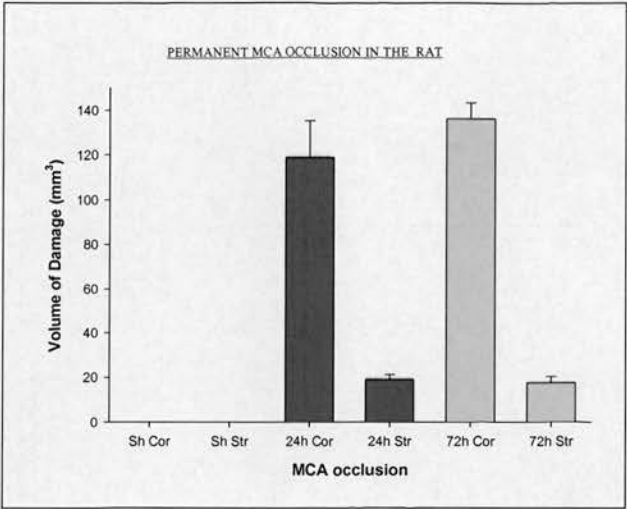


Figure 2.8: Volume of Damage observed 24 h and 72 h after onset of Permanent MCA occlusion

Thionin stained coronal brain sections (20µm) were obtained from sham-operated and permanent MCA occluded rats and the volume of damage for each treatment group measured (10 animals per treatment group). A cortical and striatal lesion was observed in both the permanent and transient stroke models. No significance difference was observed between 24 h and 72 h stroke models investigated. No damage was evident in sham animals.

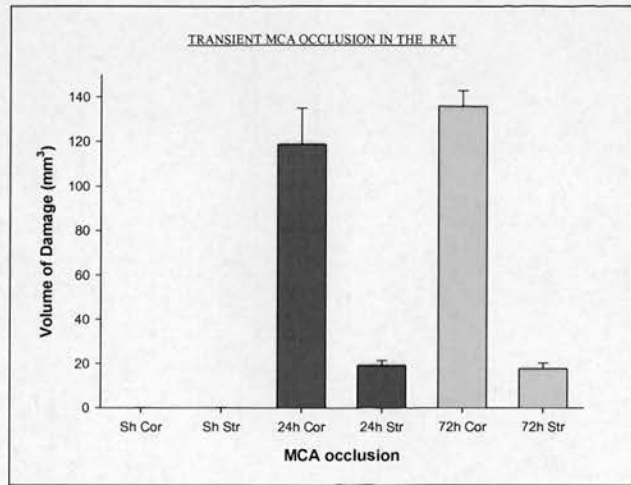


Figure 2.9: Volume of Damage observed 24 h and 72 h after onset of Transient (2 h) MCA occlusion

Thionin stained coronal brain sections (20µm) were obtained from sham-operated and transient (2 h) MCA occluded rats and the volume of damage for each treatment group measured (8 animals per group). No significant difference was observed between the time points investigated. Less variability was detected in the 72 h compared to 24 h treatment group. No significant damage could be detected in sham animals.

Twenty four hours permanent ischaemia produced a cerebral infarction in the ipsilateral cortex ($118.61 \pm 18.66 \text{ mm}^3$) and striatum ($19.02 \pm 2.53 \text{ mm}^3$), based on the loss of thionin staining. The volume of

damage observed in the cortex ($135.76 \pm 7.96 \text{ mm}^3$) and striatum ($17.53 \pm 3.19 \text{ mm}^3$) 72 h after ischaemic onset did not significantly differ from 24 h permanent ischaemia (Figure 2.8). Histological assessment following 2 h MCA occlusion with 22 h or 70 h reperfusion was investigated and compared with permanent occlusion data. Cortical ($120.01 \pm 16.10 \text{ mm}^3$) and striatal ($20.31 \pm 2.45 \text{ mm}^3$) infarcts were observed 24 h after onset of ischaemia in the transient model. Similarly, a cortical ($136.51 \pm 5.45 \text{ mm}^3$) and striatal lesion ($18.65 \pm 2.40 \text{ mm}^3$), was detected after 72 h transient (2 h) ischaemia (Figure 2.9). No significant difference in the volume of cortical and striatal damage was observed in transient and permanent models of focal ischaemia. Transient (2 h) MCA occlusion with 22 h reperfusion was chosen for preliminary investigations into pro-apoptotic Bcl-2 family response because most biochemical changes have been reported within 24 h ischaemia (Gillardon *et al.* 1995, Kitagawa *et al.* 1999).

PRO-APOPTOTIC BCL-2 FAMILY RESPONSE TO FOCAL CEREBRAL ISCHAEMIA

DNA fragmentation coupled with morphological assessment was conventionally the method used to assess apoptosis. Using this technique, early studies suggested that most biochemical changes occurred within the first 24 h of ischaemia (Tominaga *et al.* 1993, Li *et al.* 1995, Charriaut-Marlangue *et al.* 1996, Gillardon *et al.* 1996, van Lookeren Campagne *et al.* 1996, Hata *et al.* 2000, Krupinski *et al.* 2000). Moreover, return of blood flow and substrates during recirculation, accompanied by possible secondary oxidative damage might exacerbate the pro-apoptotic response (Siesjö *et al.* 1989, Hallenbeck *et al.* 1990, del Zoppo *et al.* 1994). Therefore, total cell lysates from sham-operated and MCA occluded (2 h / 22 h) rats were analysed for changes in protein expression levels of pro-apoptotic Bcl-2 family proteins Bax, Bad and Nix.

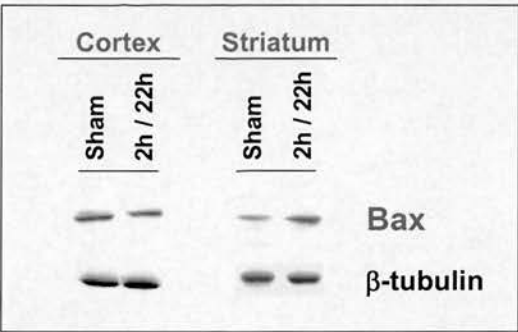


Figure 2.10: Protein expression levels of Bax after 24 h transient (2 h) MCA occlusion

Total cell lysates (30µg) from sham-operated and ischaemic rat cortex and striatum were analysed for changes in Bax protein expression (7 animals per treatment group). Bax (21 kDa) appeared endogenously expressed in both brain regions. No change in expression levels were detected 24 h after onset of transient (2 h) ischaemia. β-tubulin (50 kDa) confirmed equal loading of whole cell lysates.

Bax (Figure 2.10), total and phosphoserine residue 136 (P¹³⁶) Bad (Figure 2.11) and Nix (Figure 2.12) migrated during SDS-gel electrophoresis with apparent molecular weights of 21 kDa, 28 kDa and 48 kDa, respectively. The Nix antibody (anti-BNip3L) also detected a second immunoreactive protein with an apparent molecular weight of 30 kDa (possibly monomer of BNip3 – see Chapter 6). Each of the aforementioned proteins was endogenously expressed in the rat cortex and striatum. However the protein expression levels of these proteins did not appear to change in response to transient focal cerebral ischaemia. Cleaved caspase-3 was not detected in either brain region (Figure 2.13).

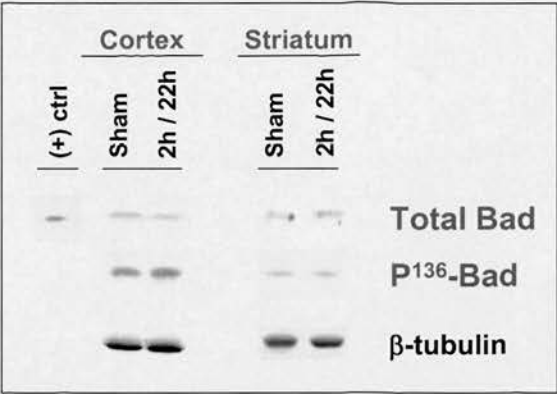


Figure 2.11: Analysis of Bad protein expression 24 h after transient (2 h) MCA occlusion

Total lysates from sham and ischaemic rat cortex and striatum were analysed for changes in Bad protein expression (7 per group). Bad (28 kDa) was endogenously expressed and phosphorylated on serine residue 136, in each treatment group. Protein expression did not change after an ischaemic insult. β-tubulin and an immunoreactive peptide confirmed equal loading and antibody specificity, respectively.

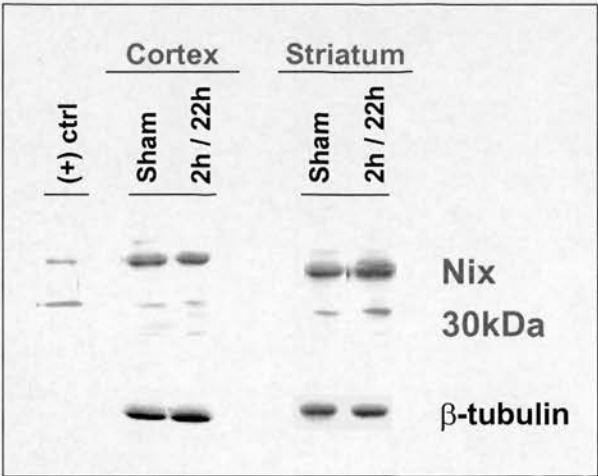


Figure 2.12: Analysis of Nix protein expression 24 h after transient (2 h) MCA occlusion

Total lysates from sham and ischaemic rat cortex and striatum were analysed for changes in Nix protein expression (7 animals per group). Two immunoreactive bands were detected with apparent molecular weights of 48 kDa (corresponding to Nix protein) and 30 kDa (possibly a monomer of Nix homologue, BNip3 – see Chapter 6). No change was detected for either protein after ischaemic insult. β-tubulin and K562 cell lysates confirmed equal loading and antibody specificity, respectively.

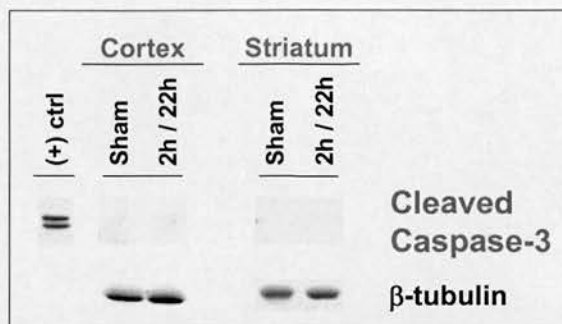


Figure 2.13: Cleaved caspase-3 protein expression 24 h after transient (2 h) MCA occlusion

Total lysates from sham and ischaemic rat cortex and striatum were analysed for changes in cleaved caspase-3 (7 animals per treatment group). Cleaved caspase-3 (19 kDa) could not be detected in either treatment group, negating a significant role for apoptosis in this rat stroke model. Equal loading and antibody specificity (+ ctrl) were confirmed using β -tubulin and apoptotic rat primary cerebellar granule cells (CGCs; *see Chapter 3*), respectively.

DISCUSSION

The aims of this chapter were to set-up a reliable stroke model which would be subsequently used to investigate the putative role of apoptotic cell death, specifically the pro-apoptotic Bcl-2 family of proteins in the pathophysiology of focal ischaemic stroke. The development of a consistently reproducible animal model of ischaemia coupled with a valid method of volumetric analysis is crucial. Evaluation of the volume of damage is one of the most amenable endpoints in focal ischaemia studies, and as such is used routinely by many stroke research groups (Isayama *et al.* 1991, Hunter *et al.*, 1995, Overgaard & Meden 2000). The only endpoint routinely accepted in neuroprotection studies is histological outcome (Recommendations for standards regarding preclinical neuroprotective and restorative drug development (Hunter *et al.* 1995, Feuerstein & Wang 2000). Precise, unbiased and reproducible assessment of infarct volume is of foremost importance (Swanson *et al.* 1990). Previous studies within this laboratory demonstrated that occlusion durations of less than 90 – 120 minutes in the rat often produced lesions with an irregular volume and pattern of damage (McCarter, 2001). In contrast, 2 h MCA occlusion was shown to produce an infarct within the MCA territory, encompassing striatal and cortical tissue (Sharkey & Butcher 1994). The standardised 72 hr post-occlusion time point for evaluation of ischaemic damage is commonly used within F.I.N.E. and other laboratories since the lesion is fully developed at this point, thereby allowing valid histological assessment and putative neuroprotection offered by various compounds. This also circumvents problems where neuroprotectants were observed to merely slow the maturation process, rather than reduce ischaemic damage *per se*. (Overgaard & Meden *et al.* 2000, Sharkey & Butcher *et al.* 1994). Most biochemical changes have been reported within 24 h permanent (Gillardon *et al.* 1996, Krupinski *et al.* 2000) and

transient ischaemia (MacManus *et al.* 1997, Charriaut-Marlangue *et al.* 1996), a time point where lesion volume is reportedly complete (Brint *et al.*, 1988, Kuge *et al.* 1995, Buchan *et al.* 1992).

Comparison of permanent and transient MCA occlusion in rat stroke models revealed no difference in the resultant volume or pattern of damage observed using thionin staining techniques. Although the lesion boundaries (encompassing the striatum and cortex) were visible by 24 h ischaemia, development of infarction within these areas was observed following 72 h ischaemia. These findings are in agreement with other studies which reported comparable lesion sizes 24 h after ischaemic onset (Bederson *et al.* 1986, Du *et al.* 1996, Lundy *et al.* 1986, Chen *et al.* 1993, Zhang *et al.* 1994). Successful MCA occlusion was achieved and confirmed by analysis of histological and neurological deficits (rotation limb weakness). Evans Blue staining confirmed correct filament placement and restoration of blood flow was confirmed in each animal. Thionin staining identified well-demarcated lesions that could be scored using the light microscope 24 h and 72 h after ischaemic onset. Initial studies established the intraluminal monofilament model as a reproducible and valid model for investigating the pathophysiology of focal cerebral ischaemia, where the volume and distribution of the damage observed both permanent and transient monofilament models was consistent with literature (Tamura *et al.* 1981, Kuge *et al.* 1995, Oliff *et al.* 1995, Herz *et al.* 1998). The reliability of the surgical models was attested by Power analysis. Using the values generated from these permanent and transient focal ischaemic stroke studies, it was calculated that 6 and 7 animals, respectively, per group would be required to detect a 50% reduction in lesion size with 80% power. These values are comparable with data obtained within F.I.N.E. laboratories (Sharkey & Butcher 1995).

Since most biochemical changes have been reported within the first 24 h of ischaemia, apoptotic responses, specifically changes in pro-apoptotic Bcl-2 family protein expression, were investigated and compared with evolution of infarction. However, no evidence was obtained for a pro-apoptotic response using this stroke model. In these initial studies, upregulation of caspase-3 cleavage products or pro-apoptotic Bcl-2 family proteins, Bax, Bad nor Nix, could not be detected following 24 h transient ischaemia. It should be noted that although numerous studies have reported DNA fragmentation in similar rat models of stroke, the relevance and reliability of such findings have been questioned. For example, excessive release of intracellular calcium, an early event following experimental stroke (Harris *et al.* 1981) is proposed to be a major mediator of both necrotic (Nasr *et al.* 2003) and apoptotic (Yu *et al.* 2002, Nasr *et al.* 2003) cell death. Calcium-activated endonuclease activation and DNA fragmentation might therefore not be the most appropriate marker of apoptosis. Several studies have provided other evidence to support apoptotic cell death following stroke: increased pro-apoptotic Bax and reduced anti-apoptotic Bcl-2 and Bcl-X_L mRNA expression levels were detected in the cortex (but not striatum) within 6 h permanent ischaemia in the rat (Gillardon *et al.* 1996). In the same study, increased mRNA expression of pro-caspase -1, -2, -3, -6 and -8 were

observed to peak 12-24 h after ischaemic onset. This was reported to co-localise with nuclear DNA fragmentation (identified using terminal-deoxynucleotidyl-transferase-mediated-dUTP nick end labelling; TUNEL) primarily in the inner boundary zone of the infarct (Li *et al.* 1995, Krupinski *et al.* 2000). Activation of the TNF- α death receptor pathway is reported to involve pro-caspase-8 cleavage and effector caspase-3 cleavage, without involvement of the mitochondria and ATP-dependent caspase-9 activation (Medema *et al.* 1997, Yang *et al.* 1998, Screaton & Xue 2000). In support of these findings, a recent study by Berti and colleagues (2002), reported a 5-fold increase in TNF- α mRNA expression levels in the injured hemisphere 3-24 h after onset of ischaemia in a rat transient (2 h) MCA occlusion model. However, in the aforementioned studies protein expression was not investigated and therefore associated alterations in the respective protein expression levels should not be assumed.

In animal models of focal ischaemia, the contribution of apoptotic cell death to damage incurred has been most directly demonstrated from the use of TUNEL staining, which detects single and double DNA strand breaks, and best supported by electron microscopy and discrimination of morphological features (Banasiak *et al.* 2000). Apoptotic cells defined this way have been found scattered, albeit in low numbers, throughout the ischaemic territory following both permanent (Linnik *et al.* 1993, Murakami *et al.* 1998) and transient (Charriaut-Marlangue *et al.* 1995, Charriaut-Marlangue *et al.* 1996, MacManus *et al.* 1997) MCA occlusion in rats and mice. Cells exhibiting these changes have been detected within a few hours of reperfusion following ischaemic periods of 1-2 hours, but peak in number after recirculation periods of 24-48 hours. However, even after extended periods of reperfusion, cells exhibiting features of apoptosis are in substantial minority in all affected regions (Li *et al.* 1995a, Asahi *et al.* 1997, Murakami *et al.* 1997). A similarly delayed appearance of apoptosis is produced with permanent MCA occlusion (Asahi *et al.* 1997), although fewer cells are apparently affected under these conditions (Murakami *et al.* 1997). Oxidative- and endonuclease-mediated damage is implicated in promoting nuclear DNA damage observed following focal cerebral ischaemia (Charriaut-Marlangue *et al.* 1995, Chopp *et al.* 1996). A study in which the type of cell loss was defined based on morphological changes found ratios between apoptotic and necrotic cells of 1:9 in focal regions, and 1:6 in the inner border zone at 48 hours following 2 hours of MCA occlusion in rats (Li *et al.* 1998). However, these figures might underestimate the number of apoptotic cells within the ischaemic lesion. For example (i) rapid removal of apoptotic cells by macrophages might occur following 24 h transient ischaemia, or (ii) apoptotic morphology might be masked by secondary necrosis, or alternatively (iii) the model in question might have produced a large primarily necrotic infarct, whereas a less severe model of MCA occlusion might exacerbate a more active type of ischaemic cell death.

An important and unresolved issue regarding pro-apoptotic involvement in ischaemic brain damage involves residual cerebral protein synthesis (CPS) and ATP levels within the focal and perifocal regions of the ischaemic brain. Cerebral ischaemia results in activation of specific genes and synthesis of their corresponding proteins, although overall protein synthesis in the post-ischaemic brain is severely suppressed (Dienel *et al.* 1980, Kogure *et al.* 1993, Li *et al.* 1994). Execution of programmed cell death is reportedly dependent on RNA, CPS, mitochondrial function (Ankarcrona *et al.* 1995), and sufficient ATP levels for the activation of caspase cascade (Leist *et al.* 1997). However, procaspase-9 is the only family member identified to date with an absolute requirement for ATP (Li *et al.* 1997, Saleh *et al.* 1999). When CPS and ATP levels in the ischaemic hemisphere of mice were measured following permanent MCA occlusion, CPS suppression was observed within 1 h ischaemia. In contrast, residual ATP levels persisted, until 24h after ischaemic onset (Hata *et al.* 2000b, Anderson & Sims 1999). Another study reported an initial reduction in ATP, followed by restoration of blood supply and ATP levels within the cortex, and less so, striatum following 24 h transient (1 h) MCA occlusion in mice (Anderson & Sims 1999). These findings suggest that, although CPS is markedly suppressed, residual ATP levels might enable activation of the apoptotic cell death program.

Several additional explanations might exist for the lack of pro-apoptotic Bcl-2 family response in the present investigation. These include: (i) low sensitivity and high background produced with Nix antibody, (ii) severity of ischaemic insult and (iii) inappropriate tools used to assess Bcl-2 family activity. In contrast, increased detection of apoptotic-like markers have been reported following a less severe ischaemic insult, such as 30 min (Du *et al.* 1996) and 1 h (Charriaut-Marlangue *et al.* 1996) MCA occlusion in rats. Therefore, based on available data in animal studies, investigation and characterisation of the Bcl-2 family response to less severe focal ischaemic insults may be warranted. Second, although some pro-apoptotic Bcl-2 family proteins, including Bax, have been observed to increase in response to an apoptotic stimuli (Wolter *et al.* 1997, McGinnis *et al.* 1999) other family members do not appear to be primarily regulated by protein expression. In contrast, most pro-apoptotic Bcl-2 family proteins identified to date, which are primarily cytosolic under control conditions, appear to share a common mechanism of action, in addition to being regulated by other post-translational modifications (e.g. phosphorylation, cleavage, conformational change etc.). *In vitro* analysis has suggested that mitochondrial accumulation of these pro-apoptotic members might be a critical event in the mitochondrial cell death pathway. Therefore, investigation of putative changes in the subcellular distribution of these proteins, rather than simply total protein expression, might provide a more useful tool to determine pro-apoptotic Bcl-2 family protein activation and the potential role of these proteins in stroke pathology.

In this chapter, several models of permanent and transient focal cerebral ischaemia were successfully set-up, where the reliability and reproducibility of the stroke models was confirmed by comparison

with data obtained within F.I.N.E. laboratories (Sharkey & Butcher 1995). Twenty four hours transient (2 h MCA occlusion) ischaemia was used to investigate putative pro-apoptotic Bcl-2 family protein changes in stroke tissue. Western blotting protocols were established for investigating pro-apoptotic Bcl-2 family proteins, Bax, Bad and Nix, as examples of 'multidomain', 'BH3-only' and 'BH3 and transmembrane domain-containing' subgroups, respectively. Preliminary studies identified that all three proteins are endogenously expressed in rat cortex and striatum. The Nix antibody recognised second immunoreactive band, representing a 30 kDa Nix-like protein. Using changes in total protein expression of cleaved caspase-3, Bax, Bad, Nix and the Nix-like proteins to assess apoptotic activity, no evidence was obtained to support a role for apoptosis, nor the Bcl-2 family, in the pathophysiology of focal ischaemic stroke. *In vitro* models of apoptosis were subsequently set-up to characterise the mechanism of action of Bax, Bad, and Nix in a less complex cell system (see Chapters 3, 4 and 5). In addition, whether a cell undergoes necrosis or apoptosis has been attributed to the severity of the initial insult. Therefore, refinement of the ischaemia models to include varied occlusion and reperfusion durations might optimize detection of Bax, Bad and Nix (see Chapters 6 and 7). Therefore, the next series of studies focussed on setting up several models of apoptosis in which to first characterise the *in vitro* mechanisms of action for each protein using a less complex cell system, prior to the perhaps more difficult *in vivo* characterisation of these proteins.

CHAPTER 3: *IN VITRO* CHARACTERISATION OF BAX

INTRODUCTION

Preliminary *in vivo* stroke studies, described in Chapter 2, failed to detect a pro-apoptotic Bcl-2 family component to the observed ischaemic pathology. This data is in agreement with several published reports that suggest ischaemic stroke models might be too severe and so favour a more necrotic-like cell death (Linnik *et al.* 1993, Lee *et al.* 2002). Since, several markers of apoptosis have been detected in the less severely damaged cortex (Gillardon *et al.* 1996, Kitagawa *et al.* 1999, Davoli *et al.* 2002) apoptosis might have been masked by large-scale necrosis in Chapter 2. Available evidence suggests that post-translation modifications, rather than protein expression *per se*, regulate Bcl-2 family protein activity (Huang and Strasser 2000). Therefore, assessment of total protein expression in the previous chapter might not have been the most appropriate measure of Bcl-2 protein activity. Chapters 3, -4 and -5 describe studies designed to characterise the mechanism of action of Bax, Bad and Nix, as examples of *multidomain*, *BH3-only (no TM domain)* and *BH3-only (with a TM domain)* pro-apoptotic Bcl-2 family members, respectively. This chapter investigated *in vitro* Bax activation in several models of apoptosis.

Pro-apoptotic Bax (Bcl-2 associated protein X) was initially described as a pro-apoptotic protein that can bind and counter the pro-survival function of the founding family member, Bcl-2 (Oltvai *et al.* 1994). Bax is widely expressed (Krajewski *et al.* 1994, Oltvai *et al.* 1993) and knockout studies revealed that it is essential for spermatogenesis (Knudson *et al.* 1995). Bax has also been implicated in anti-viral defence (Brauweiler *et al.* 1997) and as a tumour suppressor (Yin *et al.* 1997). Also, at least in some cases of human colorectal cancer, frame shift mutations were found in the gene encoding Bax (Rampino *et al.* 1997). Over-expression of Bax in mammalian cells increases the susceptibility of most cell types to apoptosis induced by external stimuli (Middleton *et al.* 2001). Essential roles of Bax in inducing apoptosis have been revealed by gene disruption of Bax alone (Knudson *et al.* 1995, Deckwerth *et al.* 1996) and of both Bax and Bak (Lindsten *et al.* 2000, Wei *et al.* 2001, Zong *et al.* 2001). Since this work was initiated, the absolute requirement for a 'multidomain' pro-apoptotic member Bax or Bak in drug-induced apoptosis has been demonstrated using *bax^{-/-}/bak^{-/-}* double knockout mice (Wei *et al.* 2001). However, one decade after the initial identification of this pro-apoptotic protein, the molecular basis by which Bax promotes cell death, more specifically how it promotes cytochrome c release from mitochondria, remains unclear.

Bax is a 21kDa protein that shares sequence homology with Bcl-2 in the Bcl-2 homology (BH) domains, BH1, BH2, BH3 and also contains a transmembrane (TM) domain. BH 1-3 domains participate in dimer formation as well as the regulation of cell death (Sato *et al.* 1994, Sedlak *et al.* 1995). The BH3 domain of Bax was reported crucial for dimer formation and cell death induction (Zha *et al.* 1996). Initial studies demonstrated that Bcl-2 over-expression prevented cell death, at least in part, by virtue of its ability to heterodimerise with and thus inhibit Bax (Oltvai *et al.* 1993). A number of biochemical functions have been identified for Bax, some of which correlate with its pro-apoptotic activity, including (i) heterodimerization with pro-apoptotic members (Han *et al.* 1996, Yin *et al.* 1994, Zha *et al.* 1996a), (ii) homodimerization with itself (Gross *et al.* 1998, Zha *et al.* 1997), (iii) release of cytochrome c from mitochondria (Jurgensmeier *et al.* 1998) and (iv) disruption of the potential across the inner mitochondrial membrane (Pastorino *et al.* 1998, Xiang *et al.* 1996). Changes in the conformation of Bax, its relocation from cytosol to mitochondria and its oligomerisation in the mitochondrial membrane are thought to be important for the initiation of apoptosis after drug-induced damage (Wolter *et al.* 1997, Ghatan *et al.* 2000, Murphy *et al.* 2000, Antonsson *et al.* 2001). Bax translocation to mitochondria has been proposed to serve as a key integration point for various apoptosis signals, where translocation has been reported in response to a wide variety of apoptotic stimuli, such as, staurosporine, dexamethasone, etoposide, IL-3 withdrawal, nitric oxide, Fas ligand, cell detachment and γ -irradiation (Hsu *et al.* 1997, Wolter *et al.* 1997, Goping 1998, Gross *et al.* 1998, Nomura *et al.* 1999, Murphy *et al.* 2000, Ghatan *et al.* 2000).

In normal cells Bax is a soluble monomeric protein present in the cytosol or loosely associated with mitochondria. Upon induction of apoptosis, the protein translocates to mitochondria, where it has been reported to form large oligomers (Wolter *et al.* 1997, Antonsson *et al.* 2001, Mikhailov *et al.* 2001). However, the molecular mechanisms regulating Bax subcellular distribution and action (s) at the OMM have been much debated. Using a set of epitope-specific antibodies to different domains of the Bax protein it was concluded that an NH₂-terminal domain of Bax (amino acids 12-24) is exposed following an apoptotic stimulus (or unless specific detergents such as TX-100 or NP-40 are added) (Hsu & Youle 1998). However the C-terminus of Bax has also been proposed to control its subcellular distribution (Nechustan *et al.* 1999). The solution structure of Bax suggests that the C-terminus occludes its BH3 domain (Suzuki *et al.* 2000) and not the N-terminus, as previously implied (Nechustan *et al.* 1999) which appears highly mobile and potentially a poor determinant of apoptotic engagement (Suzuki *et al.* 2000, Makin *et al.* 2001). Insertion of Bax into the mitochondrial membrane appears to be sufficient to induce cytochrome c release, an event that may act to amplify the apoptotic signal (Li *et al.* 1997, Jurgensmeier *et al.* 1998, Kuwana *et al.* 1998). However, the exact mechanism of Bax-mediated cytochrome c release has also been debated. Formation of a specific Bax-containing channel (Hsu & Youle *et al.* 1998, Antonsson *et al.* 2001) and 'non-specific outer membrane rupture' (Nomura *et al.* 1999, Zamzami *et al.* 2000) are two proposed models of Bax-induced cytochrome c release. Bax oligomerisation and insertion in the outer mitochondrial membrane

resulting in a “Bax-only” channel or hybrid channels formed by Bax binding to VDAC, ANT or part of an existing complex represent alternative possible mechanisms (Vander Heiden *et al.* 1999, Martinou *et al.* 2000). Thus at the molecular level, it remains unclear how Bax triggers cytochrome c release.

AIMS

The aims of this chapter were to: (a) characterise the *in vitro* pathway of activation for pro-apoptotic Bcl-2 family protein, Bax, (b) compare and contrast the molecular circuitry of Bax protein activation, with available published data and (c) investigate the spatial and temporal events in the Bax activation pathway in a given *in vitro* model of apoptosis.

MATERIALS & METHODS

(All reagents from Sigma, unless otherwise stated).

CELL CULTURE, DRUG TREATMENT & ANALYSIS OF APOPTOSIS

Staurosporine - treated human SH-SY5Y neuroblastoma cells

Human SH-SY5Y cells were grown to confluency in Dulbecco's Eagle Medium (DMEM) containing; 10% Fetal Calf Serum (FCS), 2mM L-Glutamine, 100 units/ml Penicillin and 100mg/ml Streptomycin. Apoptosis was induced by 5h / 500nM staurosporine (STS). Control cells were incubated with DMSO (1:1000).

Dexamethasone - treated mouse A1.1 monocytes

Mouse A1.1 monocytes were grown to confluency in RPMI 1640 medium containing; 5% FCS, 2mM L-Glutamine and 100units/ml Penicillin / 100mg/ml Streptomycin. Apoptosis was induced with 18h / 200nM dexamethasone (DEX). Control cells were incubated with DMSO (1:1000).

Potassium / Serum Deprivation in rat primary cerebellar granule cells

Rat primary cerebellar granule cells (CGC) were cultured in BME-cgc medium containing; 10% FCS, 2ml L-Glutamine, 100units/ml Penicillin and 100mg/ml Streptomycin. After 1 week, a serum-free media was used. Apoptosis was induced by replacing media with a KCl / FCS-free medium for 24h.

Lysates (30µg) were prepared from each treatment group and apoptosis was confirmed by detection of cleaved caspase-3 using Western blotting.

ISOLATION OF WHOLE CELL, CYTOSOLIC AND MITOCHONDRIAL FRACTIONS

Human SH-SY5Y cells and mouse A1.1 cells were treated with staurosporine (5 h/500nM) and dexamethasone (18 h/200nM) respectively. Control cells were treated with DMSO (1:1000). Collecting cells using a cell scraper stopped the experiment. Contaminating media was removed by spinning cells at 200g / 7min twice in HBSS. The resultant cell pellet was re-suspended in an extraction buffer optimized for subcellular fractionation of cells (SCF Buffer: 10mM Tris-Cl (pH 6.7), 0.25M sucrose, 1mM EGTA, 10mM KCl, 0.15mM MgCl₂, 0.1mM DTT, 1mM PMSF, 20µM lactacystin, phosphatase inhibitors (2.5mM sodium pyrophosphate, 1mM β-glycerolphosphate and 1mM sodium orthovanadate) and 10µg/mL protease cocktail inhibitor – aprotinin, pepstatin and leupeptin). Cells were homogenised using a tight-fitting glass Dounce homogeniser (Jencons), by performing 6 ‘up/down’ strokes on ice. Crude lysates were spun at 600g for 3 min to remove nuclei, insoluble material and unbroken cells. The resultant supernatant represented the whole cell lysate fraction.

Whole cell lysates from control and apoptotic cells were subjected to differential centrifugation methods and spun at 6,000g for 10 min using a benchtop centrifuge (Biofuge *fresco*, Heraeus Instruments) to separate the cell lysate into crude cytosolic (supernatant) and mitochondrial (pellet) fractions. The crude mitochondria-containing pellet was washed twice (6,000g for 10 min) with SCF buffer and the resultant enriched-mitochondrial fraction re-suspended in the SCF buffer (containing 2% CHAPS) for 30 min to facilitate mitochondrial membrane solubilisation. The crude cytosolic fraction was centrifuged at 100,000g for 30 min in an ultracentrifuge (TL-100, Beckman-Coulter). The soluble fraction was retained and represented the S100 cytosolic fraction. Samples were maintained at 4°C throughout the fractionation procedure. The purified cytosolic and enriched-mitochondrial fractions (30µg per lane) were analysed by SDS-PAGE and standard Western blotting (see Appendix C).

MTS Cell Viability Colorimetric Assay

SH-SY5Y cells were cultured in 6-well plates and treated with either STS (500nM; 0-6 h) or DMSO vehicle (1:1000). The experiment was stopped by removal of the media by aspiration. Each well was washed three times with warm HBSS and replaced with fresh culture media (1000µl). MTS reagent (200µl; *see Appendix D*) was added to each well and incubated for 1-4 h @ 37°C until sufficient colour had developed. 20µl media was removed from each well and transferred to a 96-well plate and the absorbance read at 490nm. Wells containing STS alone provided the negative control.

Analysis of Protein Expression by Subcellular Fractionation and Western Blotting

Control and apoptotic human SH-SY5Y and mouse A1.1 cells were lysed, separated into cytosolic and mitochondrial fractions using subcellular fractionation and incubated overnight at 4°C with one of the following rabbit polyclonal primary antibodies: anti-cleaved caspase-3 (1:1000; New England BioLabs); anti-BaxNT (1:500; Upstate) or anti-cleaved caspase-9 (1:1000; Cell Signalling Technology). Membranes were also incubated with mouse primary antibodies: anti-β-tubulin (1:1000), or anti-α-tubulin (1:2000) and VDAC/porin (1:2000; all Sigma) to ensure: equal loading, protein transfer and fraction purity in crude cell lysate, cytosol and mitochondrial fractions, respectively. The immunoreactive proteins were visualized by incubating with the appropriate horseradish peroxidase-coupled anti-rabbit or anti-mouse Ig secondary antibody (1:2500; Amersham Life Sciences) and visualized using enhanced chemiluminescence (ECL; Amersham Life Sciences).

CONFOCAL MICROSCOPY

Visualisation of Bax and Cytochrome C redistribution using Confocal Microscopy

SH-SY5Y cells intended for subsequent confocal microscopic analysis were grown to confluency (as above) and then re-seeded in glass-bottomed poly L-lysine coated petri dishes (Willco Wells, 35/22 mm diameter) and allowed to attach overnight in culture media. Cells were administered either STS (0-5 h/500nM) or DMSO vehicle (1:1000). The experiment was stopped by removal of the drug using aspiration, and washing the cells with warm Hank's Buffered Saline Solution (HBSS). Cells

intended for subsequent Bax staining were fixed in 3% formalin/PBS (1 min), permeabilized using ice-cold 100% methanol (1 min) and washed three times in HBSS. In contrast, cells intended for subsequent cytochrome c staining were fixed and permeabilized in 10% methanol (5 min), washed three times in HBSS. Cells were blocked using either 8% BSA/PBS or 10% normal goat serum/PBS for 1 h to minimise non-specific binding of the antibody, prior to overnight incubation in PBS with antibodies to either Bax (Bax NT: Upstate, 10 μ g/ml) or cytochrome c (1:200, Santa Cruz), respectively. Bound antibody was detected with FITC-labelled anti-rabbit immunoglobulin (1:160, Sigma). Changes in subcellular distribution of proteins Bax and cytochrome c were visualized using a confocal microscope (Radiance 2000, BioRad). FITC-labelled Bax images were given a red pseudocolour (Photoshop, version 6.0).

ELISA Assay - Quantitative Assessment of Cytochrome C Release

SH-SH5Y cells were seeded at 100,000 / well in 24-well tissue culture grade plates, and allowed to attach overnight in their appropriate culture medium. STS was added to a final concentration of 500nM for 0-5 h, and the experiment was stopped by washing the cells twice with warm HBSS. Cells were homogenised in an optimized extraction buffer (40mM HEPES, 140mM KCl, 20mM NaCl, 5mM MgCl₂, 1mM EGTA and 10 μ g/ml protease inhibitor cocktail; aprotinin, pepstatin and leupeptin), containing either digitonin alone, or digitonin and CHAPS. The plasma membrane of cells was selectively permeabilized using 3.125 μ M digitonin (Kirchhoff 2002) and the cytosolic content of cytochrome c measured against total cellular content of cytochrome c (1% CHAPS in buffer) using a colorimetric-based ELISA assay (Bender Med Systems). The cyclophilin D inhibitor, cyclosporin A (CsA) was added to a final concentration of either 3 μ M or 10 μ M for 30 min in the culture media of control (DMSO; 1:1000) and apoptotic (4 h/500nM STS) SH-SY5Y cells. The experiment was stopped by aspiration and the cytochrome c ELISA performed, as described above.

Alkali Extraction of Outer Mitochondrial Membrane Proteins

Mitochondrial fractions of control and STS (5 h / 500nM)-treated SH-SH5Y cells were obtained by subcellular fractionation using a detergent-free buffer (*see above*). According to Antonsson *et al.* (2001), mitochondria were suspended in 0.1M sodium carbonate (Na₂CO₃), pH 12, to a final concentration of 3mg/ml and incubated on ice for 20 min. At the end of the incubation period, the samples were centrifuged at 100,000 \times g for 1h. The supernatant contained proteins attached, but not inserted into the outer mitochondrial membrane (OMM). The pellet was suspended in the standard subcellular fractionation buffer (*see above*) containing 2% CHAPS, incubated on ice for 1h, sonicated, and centrifuged at 100,000 \times g for 30 min. The resultant supernatant, which contains solubilized

integral membrane proteins, was saved. Purified fractions were analysed by standard Western blotting with rabbit polyclonal anti-Bax (1:500; Upstate); anti-Tom 20 (1:200; Cell Signalling Technology) or mouse monoclonal anti-VDAC/porin (1:2000; Sigma) and visualized as previously described.

Mitotracker Red CMXRos staining to Visualize Mitochondrial Changes in Apoptotic cells

Control and STS (5 h / 500nM) – treated SH-SY5Y cells were washed 3 times with HBSS, incubated with light-sensitive MitoTracker-Red (CMXRos, Molecular Probes) (0.5 μ M / 45min @ 37°C), washed 5 times with HBSS and fixed in 3% paraformaldehyde solution for 1 min. Cells were then washed 3 times in HBSS, permeabilized in ice-cold methanol for 1 min, washed a further 3 times with HBSS and visualized by confocal microscopy (Radiance 2000, BioRad).

Analysis of the Inner Mitochondrial Membrane Potential using JC-1

JC-1 is a cationic dye that exhibits a potential dependent accumulation in mitochondria, which is indicated by a fluorescence emission shift from red (~590nm) to green (~525nm) as the mitochondria becomes depolarized. The potential-sensitive colour shift is due to concentration dependent formation of red fluorescent J-aggregates in polarized cells (Di Lisa *et al.* 1995, Smiley *et al.* 1991). Cells were treated with either STS (0-5 h/500nM) or DMSO vehicle (1:1000). Mitochondria were directly stained with JC-1 (Molecular Probes, 10 μ M for 10 min). Cells were washed three times with HBSS and changes in $\Delta\Psi_m$ visualized using a confocal microscope (Radiance 2000, BioRad).

RESULTS

Initial studies were designed to set-up several models of apoptosis, previously reported to involve Bax activation (Wolter *et al.* 1997, McGinnis *et al.* 1999). Human SH-SY5Y, mouse A1.1 and rat CGC cells were treated with staurosporine (STS; 5 h/500nM), dexamethasone (DEX; 18 h/200nM) and potassium serum deprivation (KSD; 24h) respectively. Induction of apoptosis was confirmed by Western blot analysis where an increase in the appearance of cleaved caspase-3 (17/19 kDa) was detected in cell lysates (30 μ g) from the drug-treated compared to control groups. Equal loading was confirmed using an antibody to β -tubulin (*Figure 3.1*).

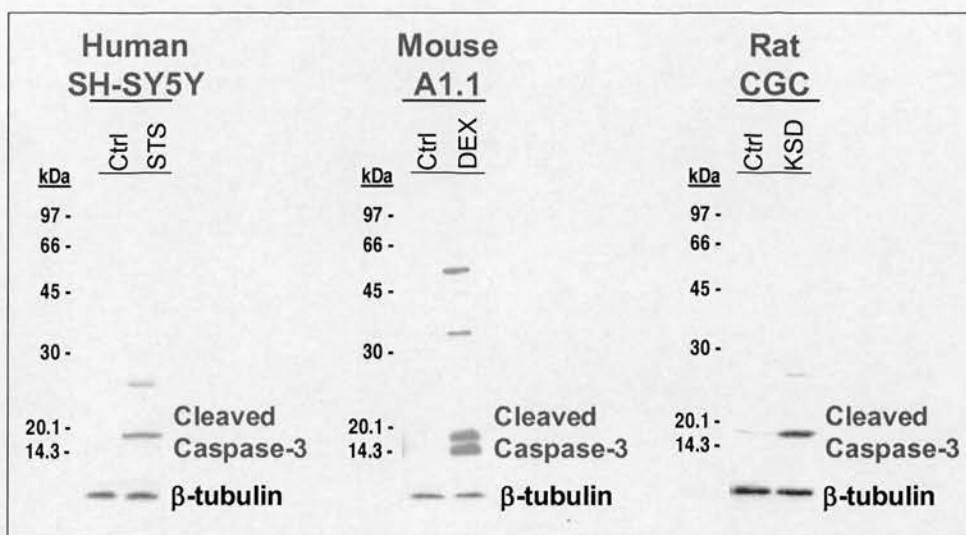


Figure 3.1: Confirmation of apoptosis in human SH-SY5Y, mouse A1.1 and rat CGC cells

Apoptosis was confirmed in staurosporine (STS; 5h/500nM), dexamethasone (DEX; 18h/200nM) and potassium serum withdrawal (KSD; 24h)-treated SH-SY5Y, A1.1 and primary CGC cells, respectively, by a marked increase in caspase-3 cleavage products (17/19 kDa). β-tubulin staining confirmed equal loading. (One representative blot of 6 individual experiments; n=6).

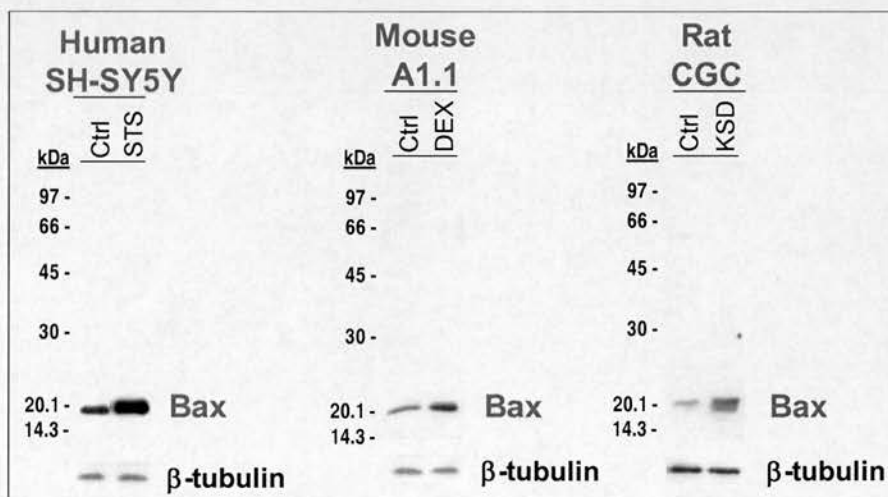


Figure 3.2: Upregulation of Bax in STS-, DEX- and KSD-treated cells

Crude lysates from control and apoptotic SH-SY-5Y, A1.1 and CGC cells were analysed for changes in Bax protein expression (n=6). A marked upregulation of Bax was observed in apoptotic compared to untreated cells. One representative blot, containing 6-10 pooled samples per treatment group.



Bax activation mechanisms remain controversial. Bax activation was therefore investigated in each cell type as a model of apoptosis. Whole cell lysates (30µg) from control and apoptotic cells were analysed for changes in Bax protein expression. Bax migrated as a 21 kDa protein, and was endogenously expressed in each cell type. However, a marked upregulation was observed in the apoptotic compared to control cells (*Figure 3.2*). Based on lack of an observed pro-apoptotic response in stroke tissue (*see Chapter 2*), it was anticipated that analysis of changes in the subcellular distribution of Bax, and other family members might be a more sensitive measure of pro-apoptotic Bcl-2 family protein activity. Subcellular fractionation was therefore optimized in cells. Cytosolic and mitochondrial fractions were prepared from control and apoptotic SH-SY5Y and A1.1 cells. These cell lines were chosen to further characterize Bax activation due to relative ease with which cells could be harvested.

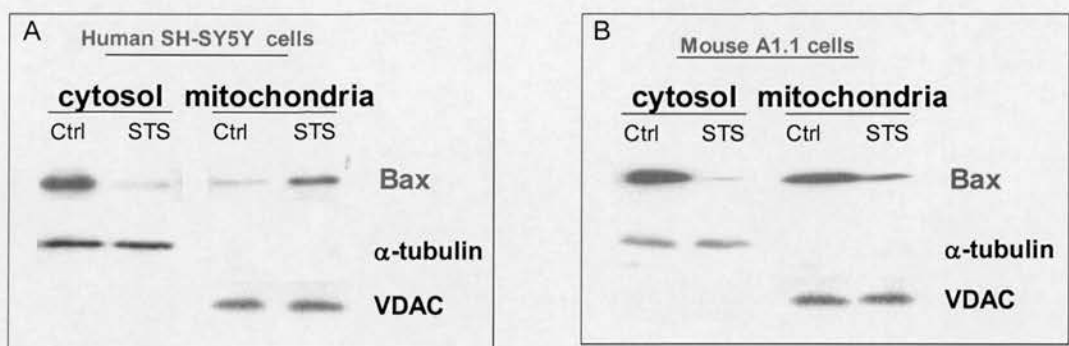


Figure 3.3: Translocation of Bax in STS- and DEX-treated cells

Crude cell lysates from control and apoptotic SH-SY5Y (A) and A1.1 cells (B) were separated into cytosolic and mitochondrial fractions and assessed for changes in the subcellular distribution of Bax (n=6). Increased mitochondrial accumulation of Bax was observed in human SH-SY5Y and less so in mouse A1.1 cells after STS and DEX treatment, respectively. Equal loading and fraction purity was confirmed with cytosolic (α-tubulin) and mitochondrial (VDAC) protein markers. One representative blot for each cell type.

Endogenous Bax was detected in both the cytosol and mitochondria of each cell type. DEX- and STS-mediated apoptosis induced re-distribution of cytosolic Bax to the mitochondria. Comparatively less Bax re-distribution was observed in A1.1 cells and this might reflect proportionately less apoptosis occurring A1.1 cells compared to SH-SY5Y cells. Equal loading and fraction purity was confirmed using antibodies raised against cytosolic (α-tubulin) and mitochondrial (VDAC) protein markers (*Figure 3.3*). SH-SY5Y cells were subsequently used to further characterise *in vitro* Bax activation, as an example of a human neuronal-like cellular response. Attempts to correlate Bax translocation with cytochrome c release from mitochondria failed, due to difficulties in obtaining cytosolic fractions free from cytochrome c in control cells.

Caspase-9 cleavage, which requires cytosolic cytochrome c, a co-factor in formation of the apoptosome (Li *et al.* 1997), was used as a surrogate marker of cytochrome c release and mitochondrial mediated cell death at a time point where Bax translocation was reported. Total cell lysates (Figure 3.4A) as well as cytosolic and mitochondrial cell fractions (Figure 3.4B) of control and apoptotic (5 h/500nM STS) SH-SY5Y cells (30µg) were incubated with a rabbit polyclonal antibody raised against cleaved caspase-9 (1:2000; Cell Signalling). Cleaved caspase-9 (37 kDa) could not be detected in control cells. However, a marked increase in cytosolic levels of cleaved caspase-9 was observed following 5 h STS treatment.

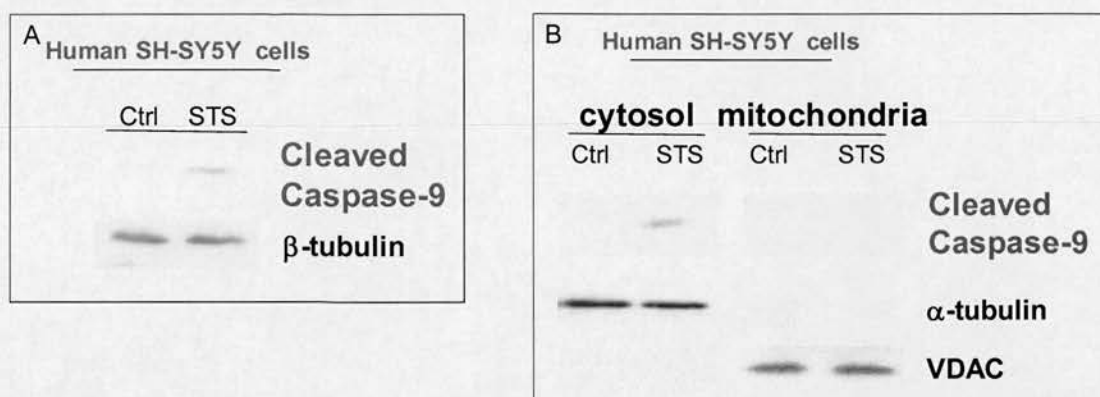


Figure 3.4: STS-induced apoptosis of SH-SY5Y cells involves apoptosome formation

Caspase-9 cleavage was investigated as a measure of mitochondrial-mediated apoptosis. Cleaved caspase-9 (37 kDa) was not detected in either fraction of untreated SH-SY5Y cells. In contrast, caspase-9 cleavage products were detected in both the whole cell (A) and cytosolic fraction (B) of STS-treated cells (Representative blots of 3 individual experiments; n=3).

An MTS cell viability assay was used to identify the optimal time point in which the cells had incurred a sub-maximal insult – with the aim of characterising cell death mechanisms upstream of the final execution phase. 5 h STS treatment was the chosen time point since approximately 90% cell death was achieved; a time where cell death mechanisms were proposed to be prevalent (Figure 3.5). These biochemical findings were confirmed under the light microscope, where SH-SY5Y cells appeared shrunken, but had not yet detached after 5 h STS treatment. Untreated SH-SY5Y cells retained their neuronal-like appearance, with a large cell body and extensive processes (Figure 3.6). Since, the Bcl-2 family were proposed to regulate mitochondrial integrity (Green & Reed 1998), STS-mediated changes in mitochondrial morphology was visualized by staining with mitochondria-specific dye, MitoTracker Red CMXRos. Mitochondria of untreated SH-SY5Y cells were observed as spherical organelles surrounding a large nuclear-like shape. Progressive rounding of cells, with retraction of processes was observed between 1-5 h STS treatment (Figure 3.7).

Time Course of STS-mediated Cell Death

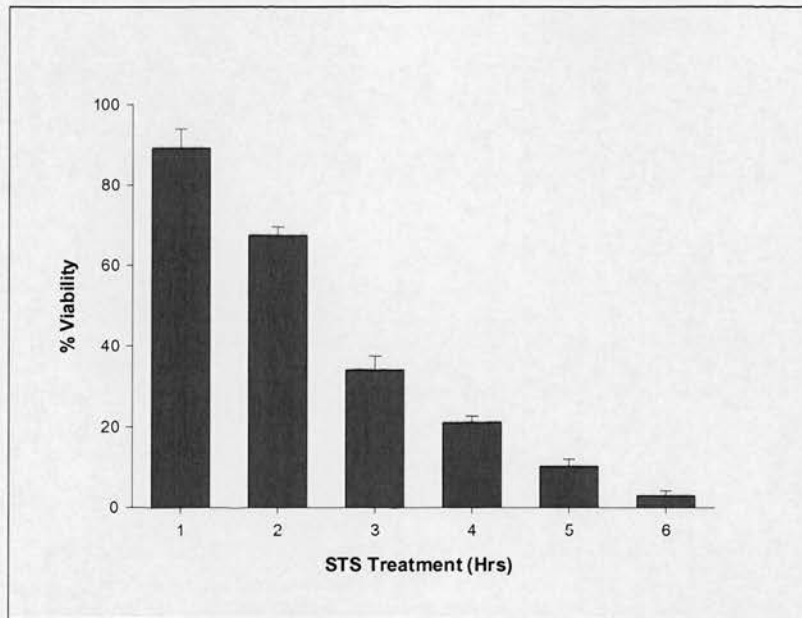


Figure 3.5: Time course of cell death in STS-treated SH-SY5Y cells

SH-SY5Y cells were treated with STS (0-6 h/500nM). Approximately 90% cell death was observed after 5h STS treatment; a time point deemed optimal for investigation of Bcl-2 family protein activation (n=3).

Human SH-SY5Y neuroblastoma cells

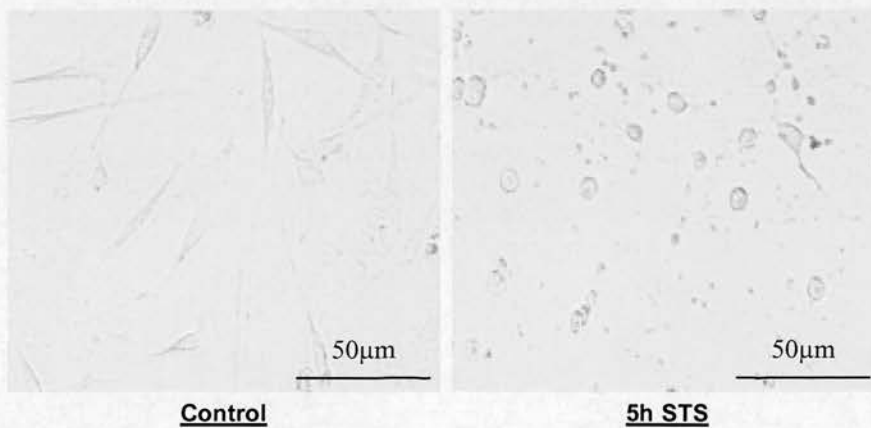


Figure 3.6: STS-mediated changes in of SH-SY5Y cell morphology

Changes in morphology were observed using a light microscope. Untreated cells displayed neuronal-like characteristics, such as large cell body with processes. In contrast, 5h STS treatment resulted in cell shrinkage, rounding of cells and retraction of processes.

Human SH-SY5Y neuroblastoma cells

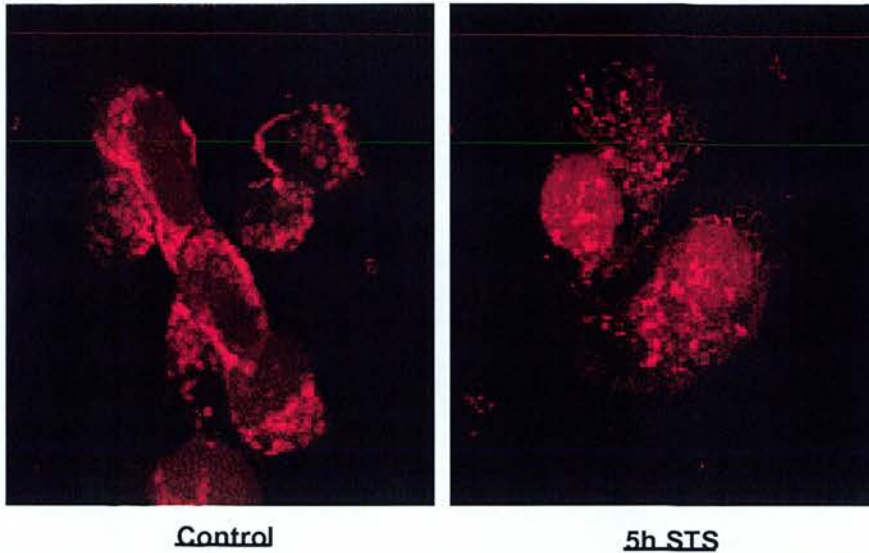


Figure 3.7: Visualisation of STS-induced mitochondrial changes using, MitoTracker CMXRos
Mitochondria of control and STS (5 h/500nM)-treated SH-SY5Y cells were visualised using mitochondria-specific dye, MitoTracker CMXRos. Unlike untreated cells which displayed a typical punctuate staining pattern, mitochondria of apoptotic cells appeared shrunken and clustered (x 40).

Apoptosis has been associated with mitochondrial swelling; however no such changes could be detected in the present study. Mitochondrial swelling, with delayed mitochondrial membrane depolarisation has been implicated in PTP opening, and is one proposed mechanism for Bax-mediated cytochrome c release (Pastorino *et al.* 1998). However, depolarisation has also been reported early during apoptosis, without detectable mitochondrial swelling (Di Giorgi *et al.* 2002). JC-1, an inner mitochondrial membrane (IMM) potential-sensitive dye, was therefore used to investigate whether mitochondrial accumulation of Bax in this model of apoptosis was associated with membrane depolarisation. Untreated SH-SY5Y cells displayed a high red:green fluorescence emission ratio, indicative of highly polarised organelles (*Figure 3.8*).

However, a progressive increase in green:red fluorescence emission ratio was observed between 1.5 h and 4 h STS treatment, indicative of STS-induced IMM depolarisation. Interestingly, onset of membrane depolarisation was apparent following 2h STS treatment and progressed until 3 h STS. Further depolarisation was not observed between 4 h and 5 h STS treatment, where apoptotic cells (5 h/500nM STS) retained partial polarity.

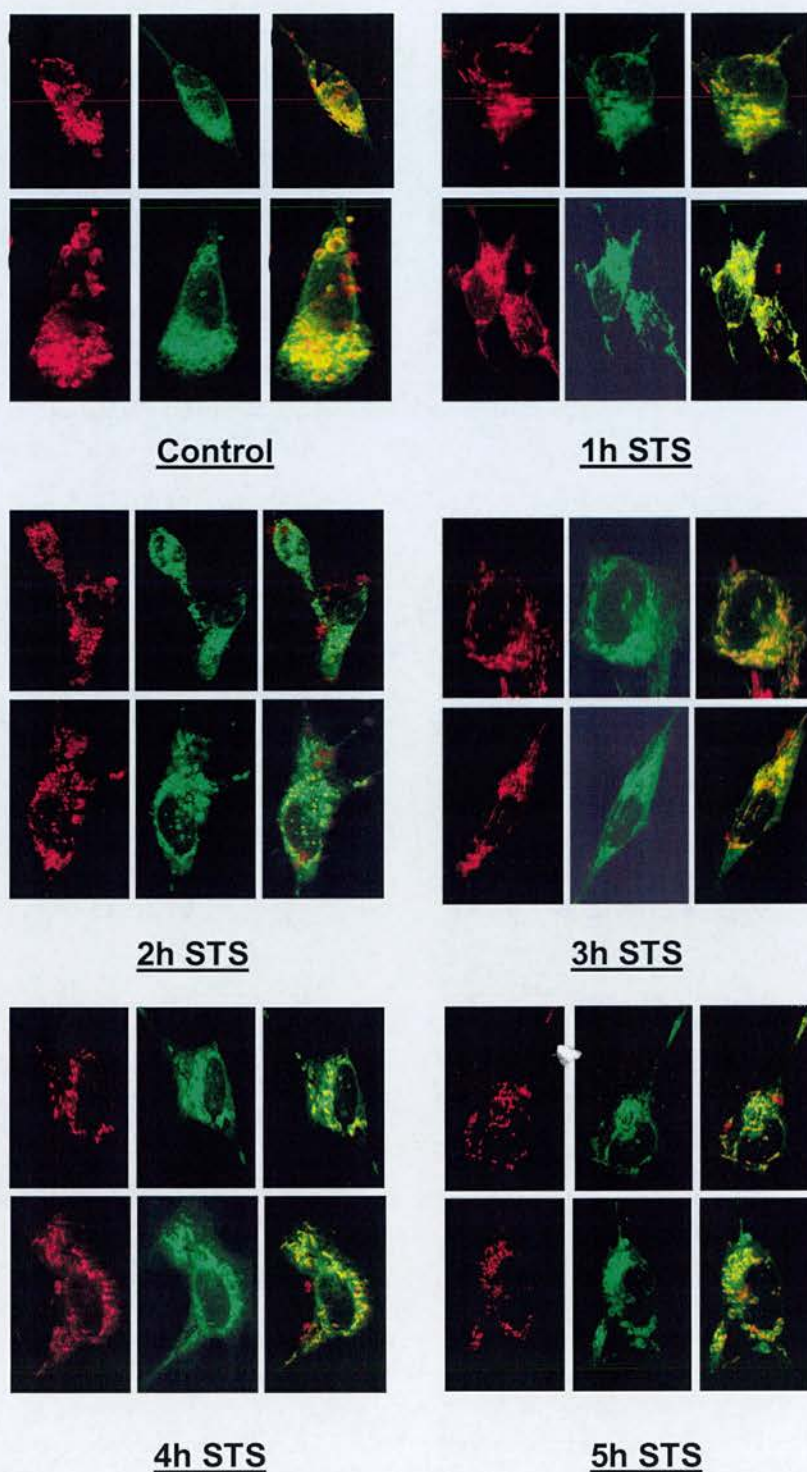


Figure 3.8: Analysis of STS-mediated changes in mitochondrial inner membrane (IMM) potential using potential-sensitive dye, JC-1

STS-mediated changes in mitochondrial membrane potential were investigated (n=3). Untreated SH-SY5Y cells displayed a high red:green fluorescence emission ratio, indicative of highly polarised healthy cells. However, a progressive increase in green:red fluorescence emission ratio was observed between 2h-5h STS treatment, indicative IMM depolarisation. Interestingly, onset of membrane depolarisation was observed following 2h STS treatment and progressed until 3h STS. Further depolarisation was not observed between 3h-5h STS. Two representative cells per time point (x 40).

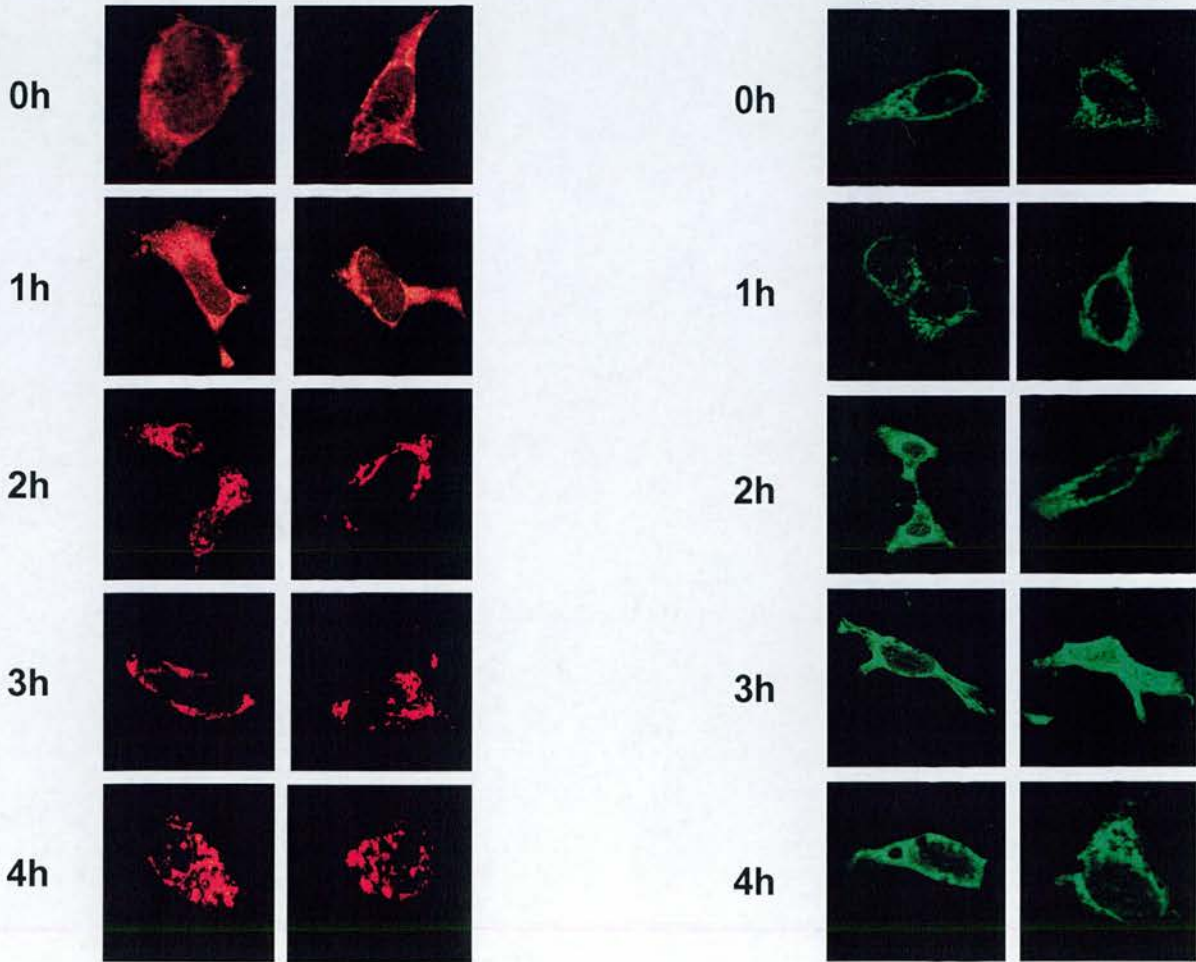
Bax has been reported to promote cytochrome c release when added directly to isolated mitochondria (Antonsson *et al.* 2000, Polster *et al.* 2003). However, attempts to investigate concomitant cytochrome c release failed using subcellular fractionation techniques. Therefore, the temporal profile of Bax and cytochrome c redistribution after receipt of an apoptotic stimulus was investigated using confocal microscopy. Endogenous Bax appeared primarily cytosolic, with minimal peri-nuclear staining (Figure 3.9A). A progressive increase in Bax re-distribution was observed and appeared complete by 3h STS. Bax staining at this latter time point appeared punctate in origin with an observed ‘clustered’ staining pattern. A similar peri-nuclear-like staining pattern, without apparent clustering, was observed for endogenous cytochrome c in untreated cells. Changes in subcellular distribution of cytochrome c were apparent following 2 h STS treatment. However, complete translocation and cytosolic accumulation could not be detected at any time point (Figure 3.9B). Even following 4h STS treatment, residual punctuate staining of cytochrome c could be detected, and therefore an ‘all or nothing’ event was not observed in this model of apoptosis. Subsequent studies identified the onset of re-distribution for each protein 1.5 h after onset of STS treatment.

A

Human SH-SY5Y neuroblastoma cells

Bax

Cytochrome C



B

Human SH-SY5Y neuroblastoma cells

Bax

Cytochrome C

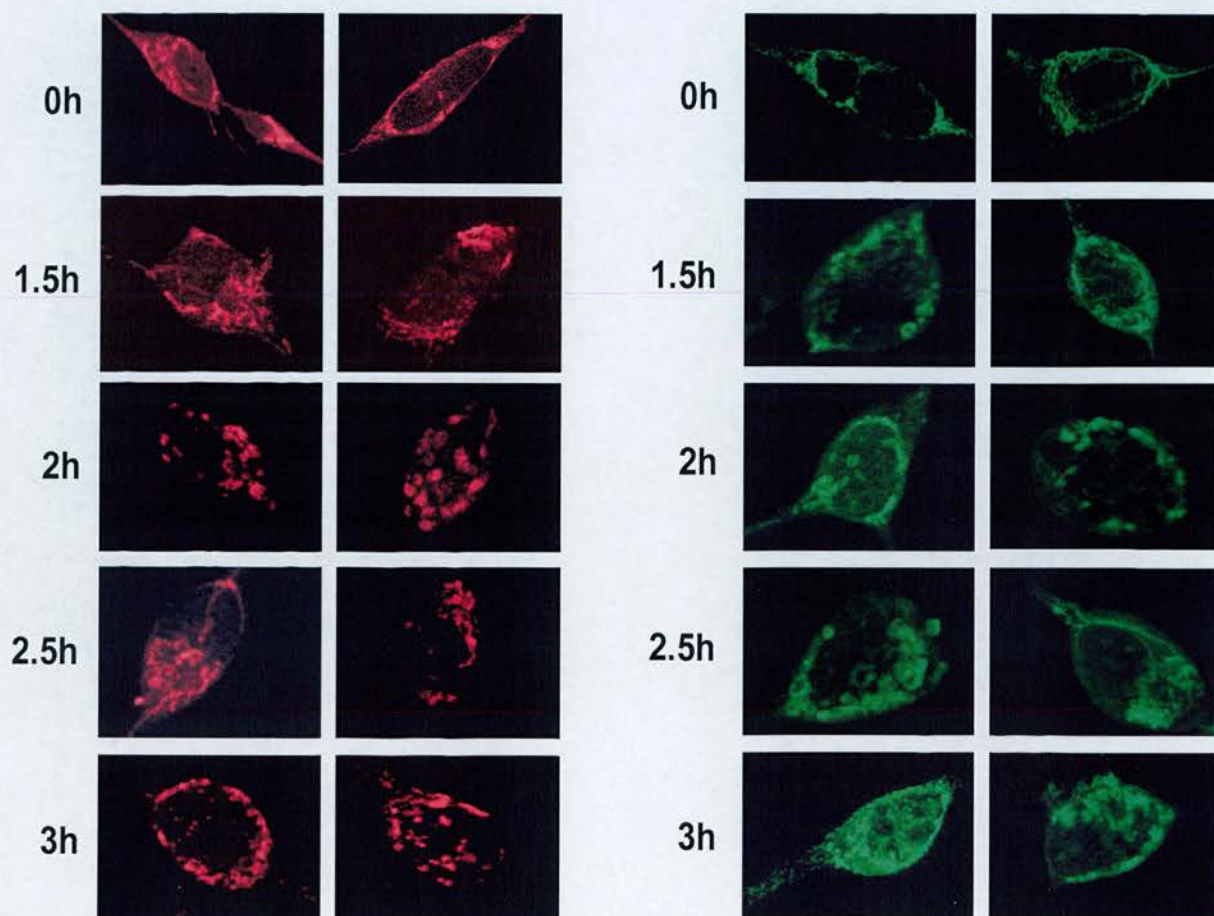


Figure 3.9: STS-mediated redistribution of Bax and cytochrome c.

The temporal profile of STS-mediated Bax and cytochrome c redistribution was investigated (n=4). In untreated cells, Bax and cytochrome c displayed diffuse and peri-nuclear staining patterns, respectively. A progressive clustering of Bax was observed throughout the time course. In contrast, no further cytochrome c release was observed after 3 h STS (A). Onset of Bax and cytochrome c redistribution was observed following 1.5h STS treatment, resulting in a peri-nuclear and diffuse staining pattern were observed, respectively (B). Two representative cells per treatment group. (x 40)

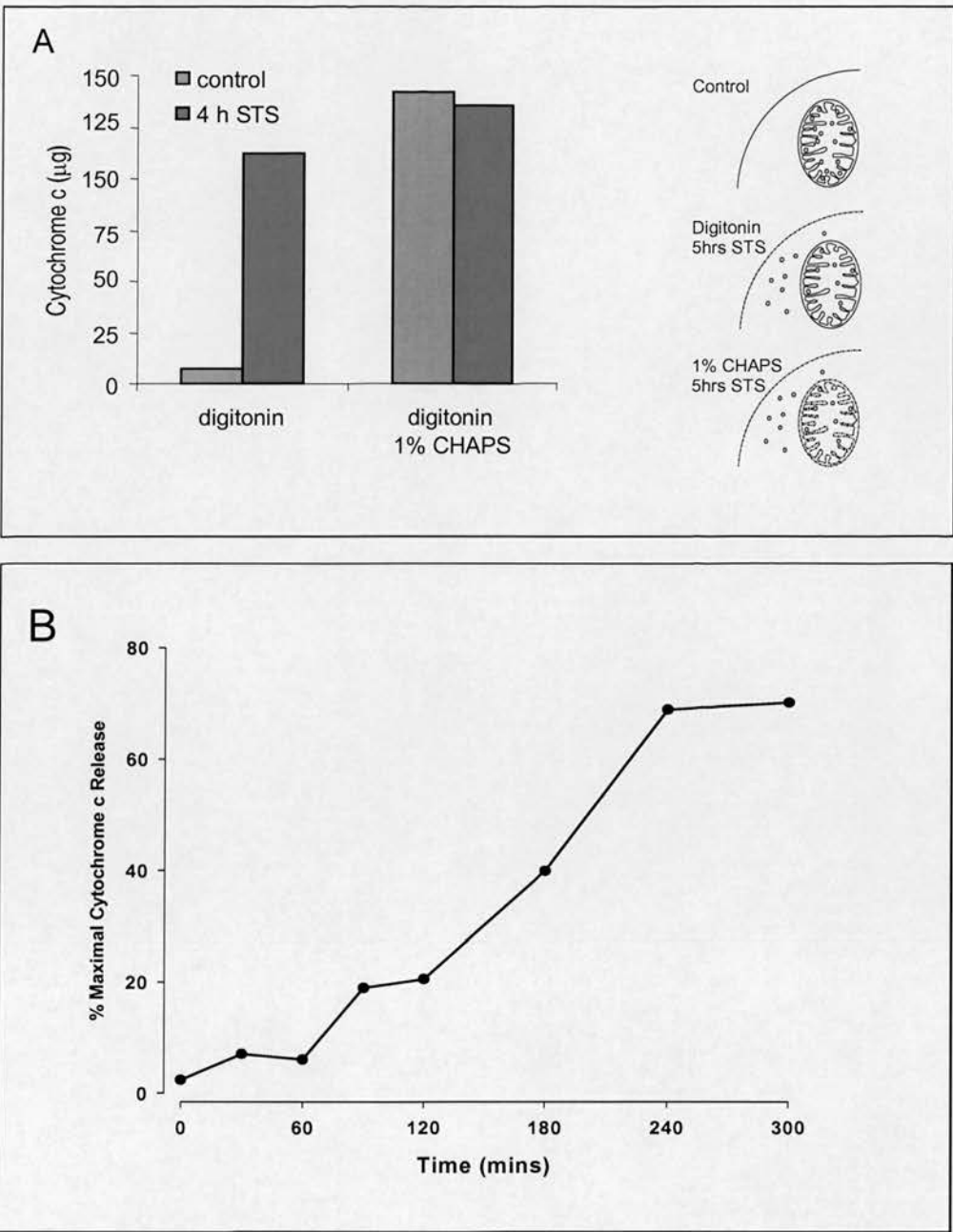


Figure 3.10: Quantitative analysis of STS-mediated cytochrome c release

The kinetics of cytochrome c release were quantified using a cytochrome c ELISA assay (n=3). Cytosolic cytochrome c was measured by selective permeabilisation of the plasma membrane using a digitonin-containing buffer, compared to complete membrane permeabilisation achieved with the addition of 1% CHAPS to the extraction buffer (A). A marked increase in cytochrome c release was detected between 1-4 h STS. Cytosolic levels of cytochrome c release were observed to plateau between 4 h and 5 h STS (B). Each time point represents the mean of 3 individual experiments.

Attempts to double label control and STS-treated SH-SY5Y cells and co-localise Bax with cytochrome c failed due to incompatibility of staining protocols. Using confocal microscopy, onset of Bax translocation was clearly observed following 1.5h STS treatment. However, onset of cytochrome

c redistribution proved difficult to determine using this method. The recent commercial availability of a cytochrome c ELISA enabled quantitative assessment of STS-mediated cytochrome c release. Selective permeabilisation of the plasma membrane was achieved using an optimized digitonin-containing extraction buffer (Kirchhoff *et al.* 2002) to measure cytosolic levels of cytochrome c in control and apoptotic (0-5 h/500nM STS) cells. Complete membrane permeabilisation was achieved using a 1% CHAPS-containing buffer (Figure 3.10A). Onset of cytochrome c release into the cytosolic fraction was observed between 1.5h and 2h STS treatment, measured as an increase in cytosolic content of cytochrome c in control ($25.4 \pm 0.97 \mu\text{g/ml}$) compared to STS-treated ($52.58 \pm 19.21 \mu\text{g/ml}$) SH-SY5Y cells. However, cytosolic levels of released cytochrome c appeared to plateau and were not significantly different between 4 h ($152.33 \pm 10.06 \mu\text{g/ml}$) and 5 h STS treatment ($152.03 \pm 23.29 \mu\text{g/ml}$). Cytosolic release of cytochrome c (μg) was expressed as a percentage of the total detergent (1% CHAPS) - induced releasable cytochrome c from the mitochondria (Figure 3.10B). The observed trends in the temporal profile of STS-mediated cytochrome c release were comparable using ELISA and confocal microscopy techniques.

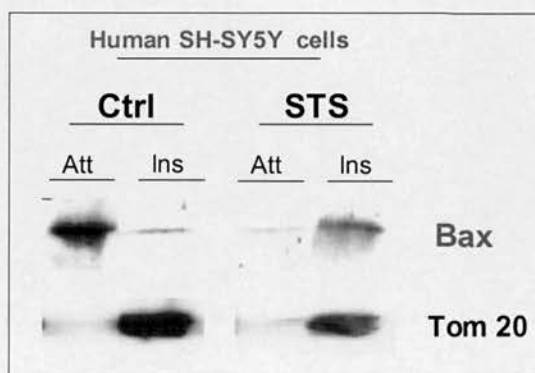


Figure 3.11: STS-mediated changes in Bax association at the OMM

The nature of Bax association at the outer mitochondrial membrane (OMM) in control and apoptotic (5h / 500nM STS) cells was determined using a Na_2CO_3 extraction method (n=3). Tom 20 (20 kDa), an integral membrane protein, was constitutively observed in the 'inserted' fraction, and confirmed fraction purity. Bax (21 kDa) was attached, but not inserted into the OMM of untreated SH-SY5Y cells. In contrast, Bax fully integrated into the OMM upon receipt of an apoptotic stimulus. One representative blot.

Controversy surrounds the mechanism of action of Bax at the mitochondria. Pro-apoptotic Bax has been proposed to form a pore, or part of a channel, which acts to permit cytochrome c release (Ishibashi *et al.* 1998, Brenner *et al.* 2000). The mechanism of Bax association at the outer mitochondrial membrane (OMM) in control and apoptotic (5 h/500nM STS) cells were therefore investigated using an alkali extraction method. Using subcellular fractionation, a small amount of Bax was observed in the mitochondrial fraction of control cells (*see Figure 3.3*). Mitochondrial associated Bax appeared 'attached' to, but not 'inserted' into the OMM of untreated SH-SY5Y cells. In contrast, apoptosis was associated with Bax insertion into the mitochondrial membrane (Figure 3.11). Tom 20

(20kDa), an integral OMM protein, was resistant to Na_2CO_3 extraction and, as expected, detected only in the 'inserted' mitochondrial fraction of both control and apoptotic cells.

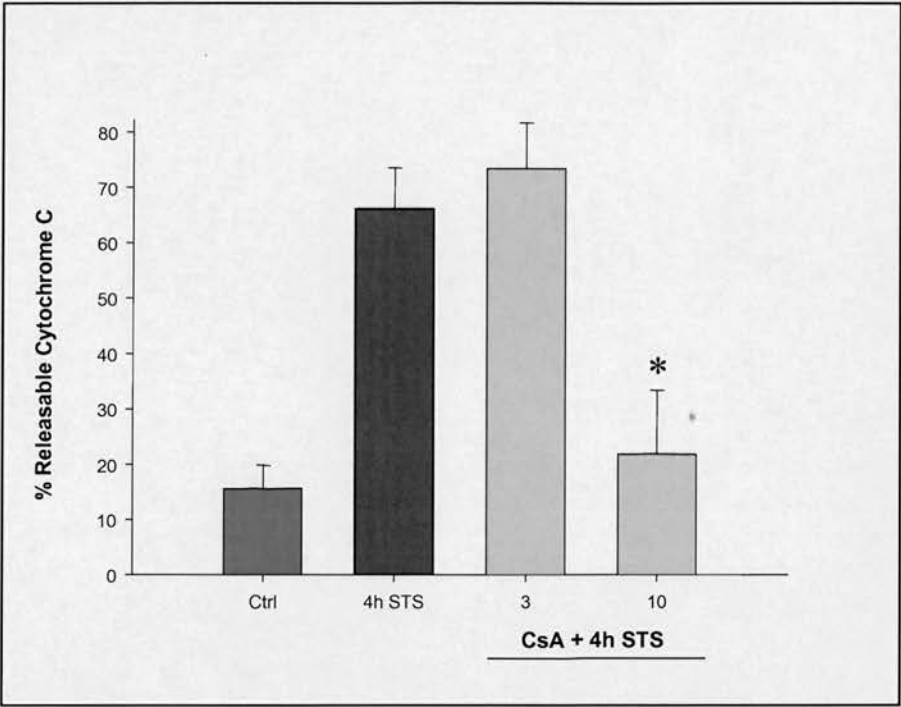


Figure 3.12: Cyclosporine A-mediated dose-dependent inhibition of cytochrome c release

Using a cytochrome c ELISA assay, the effects of PTP inhibitor, Cyclosporin A (CsA), on STS-mediated cytochrome c release were investigated (n=3). A significant and dose-dependent inhibition of cytochrome c release was observed following 10 μM CsA, but not 3 μM CsA, compared to control levels. Each time point represents the mean of 3 individual experiments.

Cumulative evidence from the aforementioned experiments suggested that STS-mediated apoptosis of SH-SY5Y cells involves Bax translocation with concomitant cytochrome c release. Furthermore, apoptosis was associated with full integration into the outer mitochondrial membrane and accompanied by mitochondrial membrane depolarisation, which has been associated with permeability transition pore (PTP) opening, but without apparent mitochondrial swelling (*see discussion*). In order to further characterise the observed mitochondrial changes associated with cytochrome c release in the model of apoptosis investigated in this chapter, the effects of PTP inhibitor, Cyclosporin A (CsA), on STS (5 h/500nM)-mediated mitochondrial release of cytochrome c were investigated using the cytochrome c ELISA. A small amount of cytochrome c was observed in the cytosol of untreated SH-SY5Y cells ($25.13 \pm 8.01 \mu\text{g/ml}$). A dose-dependent inhibition of cytochrome c release was observed following CsA treatment in the higher dose group, 10 μM CsA, ($19.10 \pm 10.84 \mu\text{g/ml}$) compared to 4 h STS treatment ($125.27 \pm 13.34 \mu\text{g/ml}$) (*Figure 3.12*). Cytosolic levels of cytochrome c following 3 μM CsA treatment were not significantly different from 4 h STS

treatment (108.90 ± 36.43 $\mu\text{g/ml}$). As predicted, neither dose of CsA had a significant effect on the detergent-induced release of cytochrome c in the control group (data not shown).

DISCUSSION

Chapter 2 detailed preliminary investigations into the pro-apoptotic Bcl-2 family response(s) to focal ischaemic stroke. Several explanations were offered for the apparent lack of any apoptotic response in this ischaemic tissue, the most pertinent being that assessment of total protein expression might not be the most sensitive method to assess pro-apoptotic Bcl-2 family activation. Changes in protein synthesis are not universal and appear to be the exception rather than the rule (Canman *et al.* 1995, Chresta *et al.* 1996). Importantly, most available data describing the mechanism of action of various Bcl-2 family proteins has been obtained using artificial systems (Oltvai *et al.* 1993, Sato *et al.* 1994, Hanada *et al.* 1995, Sedlak *et al.* 1995, Zha *et al.* 1996). Attempts to validate such findings have exploited site directed mutagenesis coupled with studies of function, often after over-expression in a variety of cellular backgrounds, cell free systems, or isolated organelles. As such, questions have been raised regarding the physiological relevance of these findings, with a growing awareness of the need for an increased knowledge of cell type-, stimulus-, and Bcl-2 family protein-specific response to apoptosis. Therefore, the aims of this chapter were to set-up several *in vitro* models of apoptosis to be used to further characterise Bax activation as well as to set-up subcellular fractionation and confocal microscopy as alternative, more suitable techniques to investigate endogenous pro-apoptotic Bcl-2 protein response(s) to apoptosis.

Several *in vitro* models of apoptosis were successfully set-up, as confirmed by increased detection of caspase-3 cleavage products (17/19 kDa). Apoptosis was induced in human SH-SY5Y neuroblastoma, mouse A1.1 monocyte and rat primary cerebellar granule cells (CGC) treated with staurosporine (STS), dexamethasone (DEX) and potassium serum withdrawal (KSD), respectively. The aforementioned models were chosen because of previously published reports demonstrating Bax upregulation and translocation in response to the apparently disparate apoptotic stimuli, in which the mechanism of action other than causing mitochondrial stress, is largely unknown (Wolter *et al.* 1997, Murphy *et al.* 1998, McGinnis *et al.* 1999). Data presented in this chapter demonstrated that endogenous Bax could be detected in the whole cell lysates of each cell type, where STS-, DEX- and KSD-induced apoptosis was associated with increased Bax expression. Although, a dexamethasone-induced increase in Bax expression was observed in the present study, this was not the case for DEX-treated chronic lymphocytic B-cells (Bellosillo *et al.* 2002); emphasising the need to characterise cell type and stimulus specific responses for each Bcl-2 family protein. A subcellular fractionation protocol was subsequently developed to investigate changes in the subcellular distribution of Bax, as a

measure of protein activity. Successful fractionation with isolation of both mitochondrial and cytosolic fractions was confirmed using VDAC (a constitutively expressed outer mitochondrial membrane protein) and α -tubulin (a constitutively expressed cytosolic protein), respectively.

Human SH-SY5Y and mouse A1.1 cells were chosen to further investigate mechanisms of Bax activation. While rat primary CGCs may have been advantageous when comparing *in vitro* and *in vivo* Bax activation, the aforementioned cell lines were chosen because of the suitable number of cells that could be grown for each experiment. Preparations from these homogeneous cell lines were consistent and this increased the reproducibility of the data obtained. Nevertheless, it should be noted that these experiments were conducted in immortalised cell lines and so care should be taken when extrapolating this *in vitro* data and comparing with *in vivo* mechanisms. In agreement with previous findings endogenous Bax was observed in the cytosolic, and to a lesser extent, mitochondrial fraction of both SH-SY5Y and A1.1 cells (Wolter *et al.* 1997, Murphy *et al.* 1998). Apoptosis induced by STS and DEX resulted in translocation of cytosolic Bax to the mitochondrial fraction. However, caution should be observed when interpreting such data. Anoikis (cell detachment)-mediated changes in both the conformation and subcellular distribution of Bax have been observed and appear to be reversible events that do not necessitate onset of apoptosis in this system (Makin *et al.* 2001). Because detachment of SH-SY5Y cells occurred (although kept to a minimum) the possibility that the above observations were partially an artefact of the experimental set-up cannot be excluded. Moreover, even intact adherent SH-SY5Y human neuroblastoma cells have been reported to express Bax dimers/complexes at the mitochondria, regardless of cell fate (Makin *et al.* 2001). Therefore, as with other markers of activation, mitochondrial accumulation of Bax alone does not necessitate Bax-mediated cell death, and it is likely other components of this multi-step cell death process are required. Despite these caveats, SH-SY5Y cells were chosen to further characterise *in vitro* mechanisms of Bax activation, because of the relative ease to perform immunocytochemical analysis on attached cells compared with cell suspensions as well as these cells more closely reflecting human neuronal-like mechanisms. Confocal microscopy was used as an additional method in which to investigate Bax activation. This technique proved advantageous in that substantially fewer cells were required to perform the experiments and avoided any anoikis-mediated changes in Bax subcellular distribution.

Several studies point to Bax as a trigger for cytochrome c release (Eskes *et al.* 1998, Jurgensmeier *et al.* 1998, Rosse *et al.* 1998, Finucane *et al.* 1999). However, the exact mechanism by which Bax, and related proteins mediate cytochrome c release is a matter of much controversy. In cells exposed to certain apoptotic stimuli, Bax, or the BH3 domain-only protein, Bid, translocates to the mitochondria (Wolter *et al.* 1997, Luo *et al.* 1998, Desagher *et al.* 1999) to initiate cytochrome c release (Jurgensmeier *et al.* 1998, Li *et al.* 1998, Luo *et al.* 1998, Narita 1998, Rosse 1998). The identification of ion channel formation in synthetic lipid bilayers by some members of the Bcl-2 family, such as Bax

(Antonsson *et al.* 1997, Schlesinger *et al.* 1997), Bid (Schendel *et al.* 1999, Zamzami *et al.* 2000), Bcl-X_L (Minn *et al.* 1997), and Bcl-2 (Schendel *et al.* 1997), suggests that pro-apoptotic members of this family might interact directly with the outer mitochondrial membrane to allow efflux of cytochrome c. Confocal microscopy provided both a temporal and spatial profile of Bax and cytochrome c redistribution in STS-treated SH-SY5Y neuroblastoma cells. Although the incompatibility of staining protocols for antibodies directed towards Bax and cytochrome c prevented investigation of co-localisation of these two regulatory proteins within the same cell, such findings might be difficult to obtain due to reported rapid release of cytochrome c (within a few minutes in some cells) from the mitochondria (Goldstein *et al.* 2000). In control SH-SY5Y cells, Bax displayed a diffuse staining pattern, with some peri-nuclear staining, characteristic of a primarily cytoplasmic protein, partially localised to mitochondrial membranes. This data is consistent with subcellular fractionation data reported in the present study. Upon receipt of an apoptotic stimulus, Bax redistribution was observed between 1-1.5 h STS treatment. Bax staining became punctate with increased duration of STS treatment. In contrast, changes in cytochrome c staining from punctuate, typical of a mitochondrial-associated protein, to more diffuse were observed between 1.5 and 3 h STS treatment. Onset of translocation proved difficult to predict. Unlike previous reports which suggest cytochrome c release to be both a rapid, 'all-or-nothing' event (Goldstein *et al.* 2000), cytochrome c release in the present study displayed characteristics of a more graded and time dependent response. Alternatively, the kinetics of cytochrome c release might have been dependent on severity of insult. For example, 0.5 μ M STS was used in this study, compared to 1 μ M STS used in the study by Antonsson *et al.* (2001).

This chapter demonstrated that the ELISA assay provided a quantitative assessment of STS-induced cytochrome c release. In agreement with data obtained from confocal microscopy, a marked increase in cytosolic cytochrome c concentration was detected following 1.5 h STS treatment. Interestingly, data obtained from both subcellular fractionation and ELISA experiments demonstrated that a small proportion of cytochrome c remained mitochondrial-bound, even after 4 h STS treatment. Only approximately 70% of total mitochondrial-associated cytochrome c was released after 4h STS treatment, a time point where Bax translocation appeared complete and after which time loss of cell structure was observed. These findings are in agreement with other published data which document one-, two- or multi-step cytochrome c release (Ott *et al.* 2002, Scorrano *et al.* 2002). Considering that apoptosis is an ATP consuming process (Leist *et al.* 1997), it is plausible that the initial fraction of cytochrome c released might participate in apoptosome formation, whereas the portion of cytochrome c that remains mitochondria associated might temporarily warrant sustained ATP production (van Loo *et al.* 2002). It has been proposed that the initially released pool of cytochrome c might be the soluble fraction from the inter-membrane space, whereas the second pool might comprise the fraction more tightly associated within the inner membrane (Ott *et al.* 2002, Scorrano *et al.* 2002). In favour of this proposal, in the present study the observed cytochrome c release following detergent solubilisation of

mitochondrial membranes was of greater magnitude than that observed following 5 h STS treatment. Therefore, the 'point-of-no-return' might not be an abrupt phenomenon as previously proposed, but rather a process accumulating in the decisive disruption of the mitochondrial membrane potential.

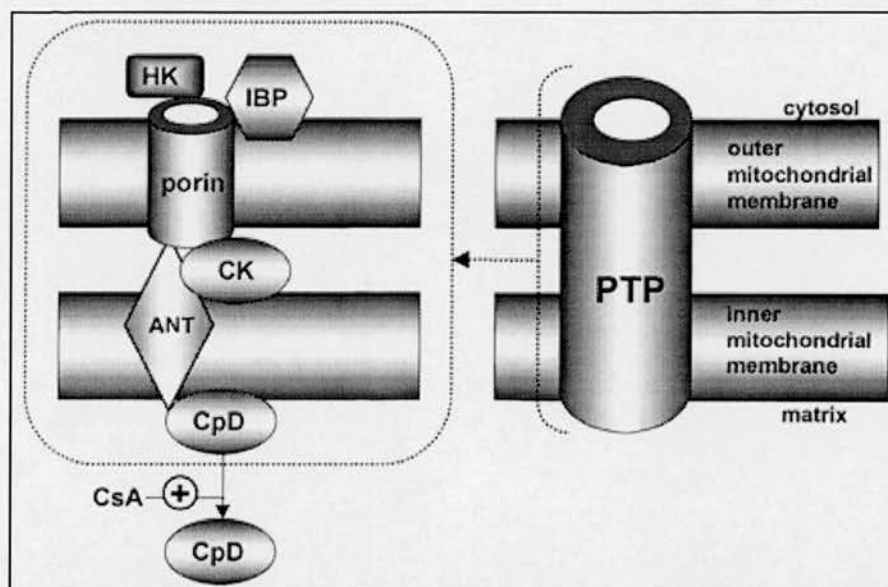


Figure 3.13: The mitochondrial permeability transition pore (Picture from Szewczyk and Wojtczak 2002).

The mitochondrial membrane permeability pore (PTP) spans the inner and outer mitochondrial membrane and is comprised of: hexokinase (HK), peripheral benzodiazepine receptor (IBP), voltage-dependent anion channel (porin /VDAC), creatine kinase (CK), adenine nucleotide translocase (ANT) and cyclophilin D (CpD). PTP opening has been implicated in some models of apoptosis and proposed to act as cytochrome c permeable channel during apoptotic cell death. PTP opening has been reported in animal models of focal cerebral ischaemia, where reduced infarct volumes were observed following treatment with cyclosporine A (CsA).

The precise mechanism(s) by which pro-apoptotic Bcl-2 family proteins induce mitochondrial damage remains an unresolved and controversial issue, with evidence for and against several independent schools of thought. Several models prevail and include: (i) cytochrome c is released as a result of the opening of the permeability transition pore (PTP), a large poorly characterised protein complex at sites between the inner and outer mitochondrial membranes (Zamzami *et al.* 2001; Figure 3.13); (ii) an alternative model favours direct Bcl-2 family interaction with components of the PTP (Martinou *et al.* 2001, Zamzami *et al.* 2001), primarily ANT (Marzo *et al.* 1998) and/or VDAC (Shimizu *et al.* 1999), or (iii) Bax might form tetramers in the OMM capable of releasing cytochrome c, and possibly other intermembrane space proteins (Antonsson *et al.* 2000, Saito *et al.* 2000). Although, data presented in this chapter demonstrated that STS-mediated cytochrome c release was associated with early mitochondrial membrane depolarisation, mitochondrial swelling was not observed, arguing against a role for PTP opening. However, it remains to be determined whether such channels are

formed *in vivo* and if indeed these can permeabilize the OMM to cytochrome c, AIF and Smac/DIABLO.

In an elegant study by Antonsson and colleagues, alkali extraction methods were used to demonstrate STS- and UV irradiation-mediated changes in Bax association at the OMM of HeLa cells (Antonsson *et al.* 2001). These initial findings supported a role for pore formation in mediating cytochrome c release, at least following STS treatment of HeLa cells. The present study was designed to investigate whether STS-induce Bax insertion also occurred in human SH-SY5Y neuroblastoma cells. Bax was attached, but not inserted into the OMM of untreated cells. In agreement with published studies (Antonsson *et al.* 2001), apoptosis induced mitochondrial accumulation of Bax was associated with concomitant Bax insertion into the OMM. Fraction purity was confirmed by Tom 20, an integral OMM protein and component of the mitochondrial import machinery. Studies using artificial membranes suggest that the concentration of exogenous Bax added to planar lipid membranes dictates the mechanism of cytochrome c release. For example, high doses of Bax (>100nM) either form non-specific channels with conductances up to 2nS (Antonsson *et al.* 1997, Brenner *et al.* 2000, Schlesinger *et al.* 1997, Shimizu *et al.* 2000) or non-specifically destabilise the membrane by diminishing its surface tension (Basanez *et al.* 1999). In contrast, at lower doses (1nM), Bax only acts on membranes also containing the PTP constituent ANT (Brenner *et al.* 2000). These findings support two independent roles for Bax at mitochondria. However, whether such mechanisms exist for endogenous Bax remains to be investigated. Additionally, it is possible that different kinetics exist for STS-mediated apoptosis in SH-SY5Y and HeLa cells. Also, the various mediators of membrane permeabilisation (e.g. VDAC) might be sequentially recruited depending on the concentration of mitochondrial-associated Bax. In this scenario, Bax-selective pore formation might mediate cytochrome c release following a mild apoptotic insult. With increased severity of insult, components of the PTP might be recruited to facilitate the formation of a larger pore and to potentiate cytochrome c release. Data obtained in the present study supports such a role for Bax, in as much as, a relatively severe apoptotic insult (5 h/500nM STS) was associated with mitochondrial membrane depolarisation, indicative of PTP-mediated cytochrome c release. In contrast to several research groups which support a role for PTP opening in Bax-mediated cytochrome c release (Marzo *et al.* 1998, Shimizu *et al.* 1999), others have failed to demonstrate such interactions using cross-linking experiments (Eskes *et al.* 2000) or by immunoprecipitation (Antonsson *et al.* 2001). More recently, a channel with similar electrophysiological properties to the proposed Bax/Bak channel was identified in the outer mitochondrial membrane (Pavlov *et al.* 2001). However, this novel channel was reported to have a pore diameter significantly larger than that of the Bax/Bak channel allowing significant diffusion of proteins larger than cytochrome c, such as AIF and Smac/DIABLO which behaves as a ~100kDa dimer (Chai *et al.* 2000) and might therefore support a role for Bax-induced release of other intermembrane space proteins during apoptosis. Interestingly, STS-mediated Bax translocation was recently reported with concomitant cytochrome c and Smac/DIABLO release in MCF-7 cells

(Luetjens *et al.* 2001). Although Bax was shown to play a decisive role in activation of this novel channel, it was proposed that other additional OMM proteins (e.g. VDAC) likely participate (Pavlov *et al.* 2001). Bax has also been reported to interact with VDAC to create a giant cytochrome c-permeable pore in artificial membranes (Shimizu *et al.* 2000). Moreover, physical interactions between Bax or Bcl-2 and VDAC and ANT have been detected using the yeast-two-hybrid system and co-immunoprecipitation assays (Marzo *et al.* 1998, Shimizu *et al.* 1999).

Subsequent studies were therefore performed to investigate whether or not STS-induced Bax translocation and insertion into the OMM detailed in this chapter, was associated with inner mitochondrial membrane (IMM) depolarisation and/or mitochondrial swelling. Membrane potential-sensitive dyes, MitoTracker CMXRos and JC-1, were used to further elucidate *in vitro* Bax activation, specifically the mechanism of STS-mediated membrane permeabilisation and cytochrome c release. A progressive STS-mediated change in mitochondrial morphology was observed when staining with both of the dyes. MitoTracker-stained mitochondria of control cells displayed a typical peri-nuclear staining pattern, where mitochondria appeared spherical. In contrast, 5 h STS treatment induced clustering and apparent shrinking of mitochondria. Whether mitochondrial swelling promotes, or is the result of cytochrome c release remains unresolved. Bax has been reported to promote cytochrome c release in the absence of detectable mitochondrial swelling (Eskes *et al.* 1998, Jurgensmeier *et al.* 1998, Finucane *et al.* 1999, Kluck *et al.* 1999) or loss of mitochondrial membrane potential (Kluck 1997, Yang 1997, Bossy-Wetzel *et al.* 1998, Eskes *et al.* 1998, Finucane *et al.* 1999, Goldstein *et al.* 2000). The IMM potential sensitive dye, JC-1, was used to characterise the temporal profile of STS-mediated changes in $\Delta\psi_m$. A progressive, and not delayed, STS-mediated $\Delta\psi_m$ loss was observed. Interestingly, when compared with the temporal profile of both Bax and cytochrome c re-distribution, onset of $\Delta\psi_m$ loss was likewise detected between 2h and 3h STS treatment. Because permeability transition (PT) is invariably associated with a loss of $\Delta\psi_m$, these data support a role for STS-mediated cytochrome c, at least partially, dependent of PT. These data agree with a previous study which used Bax over-expression to characterise cytochrome c release with concomitant induction of the permeability transition (Martinou *et al.* 1999). The polarity of SH-SY5Y cells investigated in this chapter did not appear to change between 3h and 5h STS treatment (similar to findings with cytochrome c), where mitochondria displayed characteristics of being partially polarised. As mentioned above, residual mitochondrial membrane potential as well as mitochondrial-bound cytochrome c release might enable limited oxidative phosphorylation to ensure ATP generation for the energy-consuming formation of the apoptosome and cleavage of procaspase-9 (Li *et al.* 1997, Shi *et al.* 2002, Zou *et al.* 2003). These data contradict findings where addition of recombinant Bax to isolated mitochondria induced cytochrome c release without apparent $\Delta\psi_m$ collapse (Jurgensmeier *et al.* 1998). In addition, electron microscopy of these cells revealed that the mitochondrial structure did not change throughout the entire process of cytochrome c re-distribution (von Ahsen *et al.* 2000). These results are clearly incompatible with mechanisms for cytochrome c release based on

permeability transition (as observed in the present study) and mitochondrial swelling. However, other investigators have questioned the incautious use of potential-sensitive dyes (Metivier *et al.* 1998). For example, artefacts can arise because of the self-quenching of some dyes. Also with whole cells, the plasma membrane potential can influence the amount of dye taken up into the cell. The potential sensitive dye, JC-1, used in the present study was however chosen for its reported lack of self-quenching.

Bax has also been shown to increase pore formation in chemically defined model membranes containing either ANT or VDAC (Brenner *et al.* 2000, Marzo *et al.* 1998, Shimizu *et al.* 1999, Shimizu *et al.* 2000). Similarly, rapid over-expression of Bax has been reported to induce a type of cell death, which is efficiently blocked, by a combination of CsA and aristocholic acid (phospholipase A2 inhibitor) (Pastorino *et al.* 1998), thought to synergistically act on the PTP (Zoratti and Szabo 1995). In contrast, another study demonstrated that transient over-expression of Bax can kill COS cells independently of CsA (Eskes *et al.* 1998). Several independent groups have reported that Bax, when added to purified mitochondria, causes cytochrome c release and $\Delta\psi_m$ dissipation, and that this effect is suppressed by the PTP inhibitors N-methyl-4-Val-CsA (CsA) and bongkreikic acid (Jurgensmeier *et al.* 1998, Marzo *et al.* 1998, Narita *et al.* 1998, Pastorino *et al.* 1999). Although, data presented in this chapter demonstrated that STS-mediated cytochrome c release was associated with early mitochondrial membrane depolarisation, mitochondrial swelling was not observed, arguing against a role for PTP opening (Bernardi *et al.* 1996, Susin *et al.* 1999). Subsequent studies investigated the effects of CsA on STS-induced cytochrome c release. In these studies, a significant dose-dependent inhibition of cytochrome c release from STS-treated mitochondria was observed, supporting a role for the PTP, or components of the PTP, in this *in vitro* model of apoptosis.

The data presented in this chapter reinforces the need to investigate the mechanism of action of Bax (and other family members) following different stimuli and in different cell types. Variability in published data, of which most was obtained using artificial conditions, is likely due to the variability in experimental models used to characterise Bax activation *in vitro*. While the mechanism of Bax-induced cytochrome c release was similar to that reported by Antonsson *et al.* (2001) in STS-treated HeLa cells, subtle differences were reported. The data presented suggests that, at least in STS-mediated apoptosis of human SH-SY5Y neuroblastoma cells, Bax insertion was associated with concomitant and phasic cytochrome c release, which appeared, at least partially, a PTP regulated event, but without obvious mitochondrial swelling. Subsequent studies (detailed in the next chapter) were designed to characterise the mechanism (s) of action of Bad, as an example of a BH3-only Bcl-2 family protein and to investigate whether Bad could be activated in the same STS model of apoptosis, and if so, how would the mechanism of action of Bad, compare with the observed Bax activation pathway detailed in this chapter.

CHAPTER 4: *IN VITRO* CHARACTERISATION OF BAD

INTRODUCTION

Chapter 3 characterised the mechanism of action of Bax, as an example of a *multidomain* pro-apoptotic Bcl-2 family member. STS-mediated apoptosis of human SHSY-5Y neuroblastoma cells was associated with Bax translocation towards the mitochondria, Bax insertion into the OMM, dissipation of the inner mitochondrial membrane potential, cytochrome c release and caspase-3 cleavage. This chapter used the same *in vitro* model of apoptosis to investigate the mechanism of action of Bad, as an example of a BH3 domain-only pro-apoptotic member, without a transmembrane (TM) domain. Pro-apoptotic Bad was initially identified in yeast two-hybrid studies and found to interact primarily with Bcl-X_L as well as Bcl-2. Bad is a distant Bcl-2 family member, which bears only the most universally conserved amino acids within the BH1 and BH2 (Bcl-2 homology) domains (Yang *et al.* 1995). Most BH1/BH2-containing members harbour a typical hydrophobic C-terminal signal anchor sequence and are localised to intracellular membranes (Boise *et al.* 1993, Hockenberry *et al.* 1990). However, Bad lacks such a sequence, is cytosolic in healthy cells (Yang *et al.* 1995), mediates both heterodimerization and cell death via its BH3 domain (Zha *et al.* 1997), and therefore, is considered a BH3-only member. Deletion mapping and site-specific mutagenesis studies identified the BH3 domain in Bad, as a classic amphipathic helix which is critical for both its heterodimerization with Bcl-2 and Bcl-X_L and its death-promoting activity (Zha *et al.* 1997).

Bad is a member of a rapidly growing list of BH3-only proteins, which require binding and sequestration to anti-apoptotic members, rather than promoting cell death (Han *et al.* 1996, Wang *et al.* 1996). The BH3 domain of pro-apoptotic members appears critical for their interaction with anti-apoptotic members and cell death activity (Kelekar *et al.* 1998) and when substituted into Bcl-2, converts this protein into a death effector (Hunter & Parslow 1996). This is supported by the identification of numerous BH3 domain-only members (Bad, Bid, Bik, Bim, Blk, Bmf and Hrk) with death promoting activity (Wang *et al.* 1996, Zha *et al.* 1996, Tan *et al.* 1999). Deletion and mutagenesis analysis of the BH3 domain has revealed this to be the minimal death domain required for heterodimerization and the promotion of apoptosis (Wang *et al.* 1996, Otilie *et al.* 1997). The multitude of BH3-only members suggests that a network of independently regulated pathways within a cell may be selected in response to different stimuli. Although the precise mechanisms by which Bad proteins induce apoptosis remain unclear, available data supports the following model. In its unphosphorylated state, Bad forms heterodimers with anti-apoptotic proteins (primarily Bcl-X_L) inhibiting the pro-survival function of these proteins, thus promoting cell death (Yang *et al.* 1995). Zha and co-workers demonstrated that, in the presence of IL-3, Bad is hyper-phosphorylated on one or

more serine residues (Ser-112, -136, -155 or -170) and unable to associate with Bcl-X_L. Instead, Bad is sequestered by the cytosolic phosphoserine-binding protein 14-3-3 (Zha *et al.* 1996, Masters *et al.* 2001, Masters *et al.* 2003), or possibly lipid rafts (Ayllon *et al.* 2002). Phosphorylated Bad unable to interact with pro-survival Bcl-2-like members in the outer mitochondrial membranes cannot promote cell death (Zhou *et al.* 2000, Tan *et al.* 1999, Datta *et al.* 1997, Zha *et al.* 1997). Phosphatases such as calcineurin that dephosphorylate Bad also play an important role in regulating its activity (Ayllon *et al.* 2000, Wang *et al.* 1999, Chiang *et al.* 2001). Using mutational analysis techniques in several cell lines including HEK293, HeLa and COS-7 cells, Masters *et al.* (2001) determined that the serine residue 136 is both necessary and sufficient for 14-3-3 binding. Phosphorylation of serine residue 112 was also reported necessary for full 14-3-3 mediated inhibition of Bad (Zha *et al.* 1996). Furthermore, Ser-136 has been implicated as the predominant mediator of 14-3-3 interaction and sequestration of Bad from the mitochondria (Hsu *et al.* 1997). In the case of Ser-136, 14-3-3 can be recruited to the Bad complex, which directly or indirectly causes the eventual localisation of Bad to the cytosol, away from Bcl-X_L where it is considered inactive. Since Ser-155 and Ser-170 were recently identified targets of Bad phosphorylation, no antibodies were commercially available. Available antibodies to Ser-112 did not work, therefore an antibody directed against Ser-136 was used to characterise Bad activity.

Bad may be considered a bridging molecule interconnecting signal transduction pathways from extracellular survival factors with Bcl-2, the intracellular checkpoint on cell fate. In order to avoid apoptosis, cells require survival signals that depend on extracellular stimuli such as certain growth factors, cytokines or cell-matrix contact (Raff *et al.* 1992). The lipid kinase, phosphatidylinositol 3-kinase (PI3-K) plays a critical role in the intracellular signalling cascades mediated by survival factors through production of phosphoinositol phospholipid second messengers that activate downstream targets (Datta *et al.* 1999, Franke *et al.* 1997). Among the downstream effectors of PI3-K is the serine-threonine kinase Akt (or protein kinase B) which is regulated by binding to the phospholipid products (PtdIns-3,4-P2 and PtdIns-3,4,5-P3) as well as by phosphorylation through the phosphoinositide dependent kinase-1 (PDK-1) at the plasma membrane (Franke *et al.* 1997). Akt promotes cell survival and blocks apoptosis (Hemmings *et al.* 1997, Kennedy *et al.* 1999, Kulik *et al.* 1997). Akt is a general mediator of growth factor induced survival and has been shown to suppress apoptotic cell death in a number of cell types, induced by a variety of stimuli including growth factor withdrawal, cell cycle discordance, loss of cell adhesion and DNA damage (Dudek *et al.* 1997, Kauffmann-Zeh *et al.* 1997, Kulik *et al.* 1997, Zeleniaia *et al.* 2000). Several downstream targets of Akt, including pro-apoptotic Bad, glycogen synthase kinase-3, and transcription factors CREB, FKHL1 and NF- κ B have been identified as possible mechanisms by which Akt promotes cell survival and blocks apoptosis (Datta *et al.* 1997; Franke & Cantley, 1997; Khwaja *et al.* 1999). Akt activity is positively regulated by phosphorylation on residues Thr-308 and Ser-473 downstream of PI3-K (Burgering & Coffey 1995, Datta *et al.* 1997, Dudek *et al.* 1997, Kandel & Hay 1999). Akt phosphorylates Bad at Ser-136, which

is critical for sequestration to 14-3-3 (Lizcano *et al.* 2000, Tan *et al.* 2000, Masters *et al.* 2001). Most published data detailing the molecular circuitry of Bad employed either over-expression / mutagenesis techniques or used artificial systems, such as isolated organelles or lipid bilayers, to characterise Bad activity. While invaluable in further characterisation of this pro-apoptotic protein, such events do not necessarily occur under physiological conditions, or indeed *in vivo*. Therefore, it was necessary to characterise the *in vitro* activation mechanisms of endogenous Bad, with the aim of comparing such a mechanism of action with other pro-apoptotic Bcl-2 family proteins.

AIMS

The aims of this chapter were to: (a) identify a suitable model of apoptosis which involves Bad activation (b) characterise the *in vitro* pathway of activation for BH3-only pro-apoptotic Bcl-2 family protein Bad, (c) compare and contrast results with published data.

MATERIALS & METHODS

Subcellular fractionation and alkali extraction methods with subsequent Western blotting were used to investigate putative apoptosis-induced changes in Bad protein expression. Protocols for these techniques were described in detail in Chapter 3. However, experimental conditions specific to this study will be described below.

Analysis of Protein Expression by Subcellular Fractionation and Western blotting

Briefly, control and apoptotic SHSY-5Y (5 h/500nM) cells were lysed, separated into cytosolic and enriched mitochondrial fractions, then analysed by Western blotting. Rabbit polyclonal antibodies used were: anti-Bad (1:1000; which did not discriminate between phosphorylation status of Bad); anti-phosphoserine¹³⁶ Bad (1:1000); anti-Akt (1:1000) and anti-phosphoserine⁴⁷³ Akt (1:1000; all Cell Signalling Technology). Mouse monoclonal antibodies raised against β -tubulin (1:1000), α -tubulin (1:2000) or VDAC (1:2000; all Sigma) were used to ensure equal loading, protein transfer and fraction purity in crude cell lysates, cytosol and mitochondrial fractions, respectively. The immunoreactive proteins were visualized using the appropriate horseradish peroxidase-coupled anti-

rabbit or anti-mouse Ig secondary antibody (1:2500, Amersham Life Sciences) and enhanced chemiluminescence (ECL; Amersham Life Sciences).

Sodium Carbonate Extraction of Outer Mitochondrial Membrane (OMM) Proteins

As described previously, the mitochondrial pellet of control and apoptotic (5 h/500nM STS) cells were isolated into fractions containing proteins that were either 'attached' or 'inserted' into the OMM. Membranes were incubated overnight at 4°C with one of the following rabbit polyclonal antibodies: anti-Bad (1:1000) or anti-phospho⁴⁷³ Akt (1:1000; both Cell Signalling Technology). Bound antibodies were detected using anti-rabbit Ig-HRP conjugate (1:2500, Amersham Life Sciences) and enhanced chemiluminescence (ECL). (*All reagents from Sigma, unless otherwise stated*).

RESULTS

Chapter 3 described *in vitro* Bad activation in human SH-SY5Y neuroblastoma cells. This *in vitro* model of apoptosis was used in the present study to investigate whether pro-apoptotic Bad is also activated by such an apoptotic stimulus, and if so, to characterise the mechanism(s) of Bax activation. Whole cell lysates (30µg) from control and apoptotic (5 h/500nM STS) SH-SY5Y neuroblastoma cells were analysed for changes in total and phosphoserine (P)¹³⁶ Bad protein expression (*Figure 4.1*). Bad migrated as a ~28kDa protein. Total Bad protein levels did not change in response to apoptosis. However, a marked reduction in P¹³⁶ Bad was observed in apoptotic cells compared to control.

Increasing evidence supports a critical role for the mitochondrial accumulation of pro-apoptotic Bcl-2 family proteins upon receipt of an apoptotic stimulus (McGinnis *et al.* 1999). Since Bad protein expression levels did not change in the present study, subcellular fractionation experiments were carried out as an alternative means of assessing protein activity. Cytosolic and mitochondrial fractions of control and apoptotic SHSY-5Y cells were isolated and assessed for changes in the subcellular distribution of total and P¹³⁶ Bad. Total and P¹³⁶ Bad displayed a cytosolic distribution in untreated SH-SY5Y cells and mitochondrial Bad was not detected (*Figure 4.2*). However, after STS treatment, dephosphorylation of cytosolic P¹³⁶ Bad was observed with concomitant mitochondrial accumulation of total Bad. P¹³⁶ Bad was not detected in the mitochondrial fraction of either treatment group.

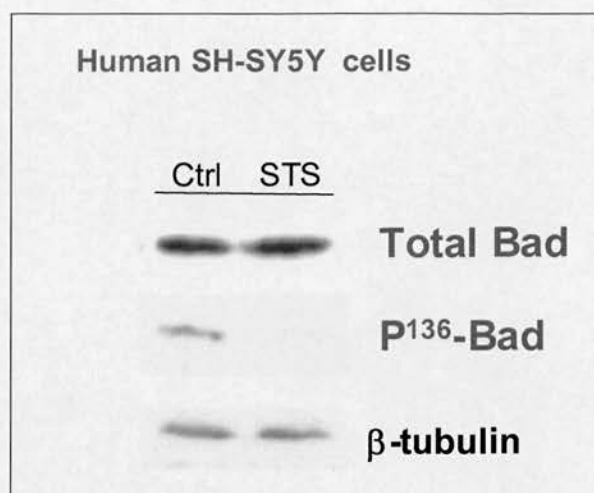


Figure 4.1: STS-mediated changes in phosphoserine136, but not total Bad

Total cell lysates from control and apoptotic (5 h/500nM STS) SH-SY5Y cells were assessed for changes in Bad (28 kDa) protein expression. Total Bad protein levels did not change in response to apoptosis. In contrast, a marked reduction in phosphoserine (P)¹³⁶ Bad immunoreactivity was observed in response to induction of apoptosis. Equal loading was confirmed using an antibody against β-tubulin. (One representative blot of 4 individual experiments; n=4).

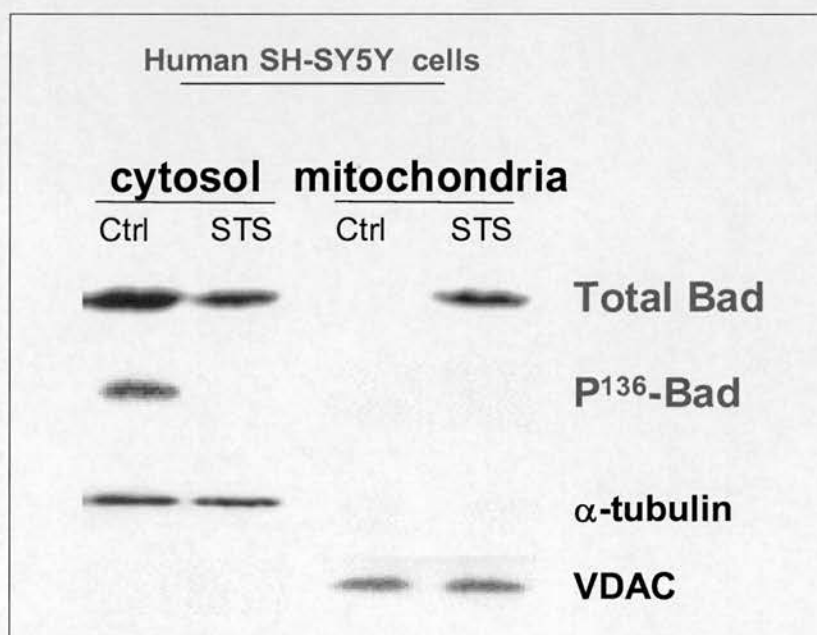


Figure 4.2: Apoptosis-induced dephosphorylation and mitochondrial accumulation of total Bad

Control and apoptotic (5 h/500nM STS) SH-SY5Y cells were isolated into cytosolic and mitochondrial fractions and assessed for changes in the subcellular distribution of Bad (n=4). Bad appeared both cytosolic and phosphorylated in untreated cells. In contrast, apoptosis was associated with dephosphorylation and an increase in mitochondrial expression of total Bad. Equal loading and fraction purity was determined using antibodies against the cytosolic marker, α-tubulin and mitochondrial marker, VDAC.

Alkali extraction methods were used in Chapter 3 to demonstrate Bax insertion into the outer mitochondrial membrane (OMM) following an apoptotic stimulus. This finding supports the proposal that TM domain-containing members might promote cytochrome c release through insertion into the OMM and pore formation. In contrast, proteins devoid of a TM domain, such as Bad, have been proposed incapable of inserting into the OMM, and likely requires binding to other proteins at the mitochondrial membrane to promote cytochrome c release (Moreau 2003). Similar alkali extraction methods were therefore used to characterise the mechanism of Bad association at the OMM following an apoptotic stimulus. Bad could not be detected, or was below levels of detection, in either mitochondrial fraction of control cells (*Figure 4.3*). In contrast, total Bad was extracted in the fraction representing ‘attached’, but not ‘inserted’ OMM proteins in apoptotic cells. Consistent with previous findings, P¹³⁶ Bad could not be detected in the mitochondrial fraction of either treatment group. Fraction purity was confirmed by detection of Tom 20 (an integral OMM protein) in the ‘inserted’ mitochondrial fraction.

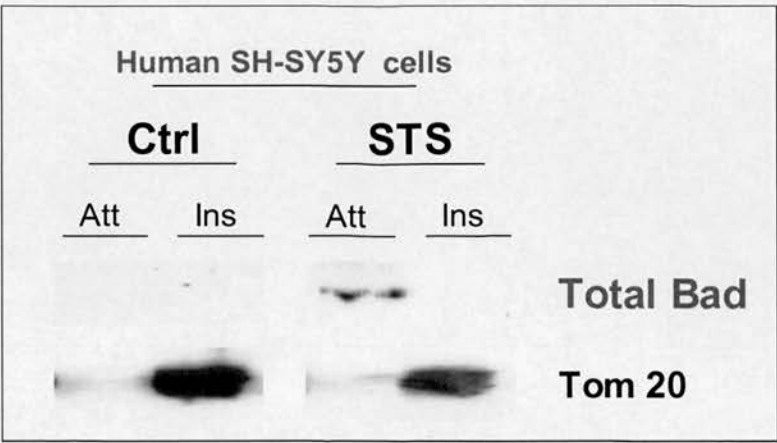


Figure 4.3: Apoptosis promotes Bad attachment, but not insertion, at the OMM of SH-SY5Y cells
Mitochondria of control and apoptotic (5 h/500nM STS) SH-SY5Y cells were isolated using an alkali extraction technique (n=3). P¹³⁶ Bad was not detected in the mitochondrial fraction of either treatment group. In contrast, total Bad appeared attached, but not inserted, into the OMM of apoptotic cells. Tom 20 (20 kDa), an integral OMM protein confirmed fraction purity.

The serine residue at position 136 of Bad is a well-characterised *in vitro* target of Akt. Akt, like Bad, is regulated by phosphorylation, where phosphorylated Akt in turn phosphorylates Bad on serine residue 136 and subsequently inactivates this pro-apoptotic Bcl-2 family protein (Datta 1997). Since a loss of phosphoserine 136 Bad immunoreactivity was detected following STS treatment, the phosphorylation status of Akt was also investigated. Whole cell lysates (30µg) from control and apoptotic SH-SY5Y cells were analysed for changes in total and P⁴⁷³ Akt protein expression. Akt migrated as a ~57 kDa protein. A marked reduction in both total and P⁴⁷³ Akt immunoreactivity was observed in apoptotic compared to control cells (*Figure 4.4*).

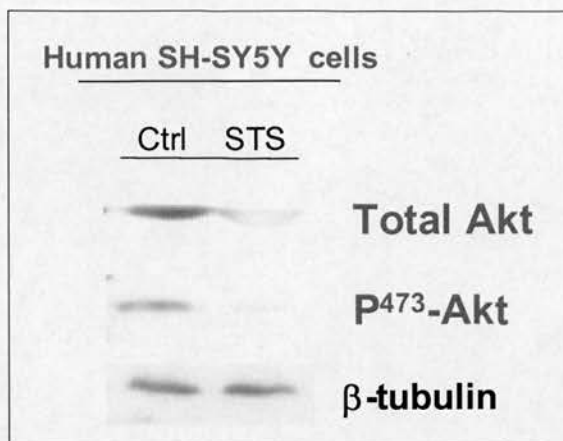


Figure 4.4: STS-mediated changes in total and phosphoserine (P)⁴⁷³ Akt

Cell lysates from control and apoptotic (5 h/500nM STS) SH-SY5Y cells were assessed for changes in Akt (57 kDa) protein expression (n=4). A marked reduction in total and P473 Akt immunoreactivity was observed in apoptotic compared to control cells. β-tubulin confirmed fraction purity.

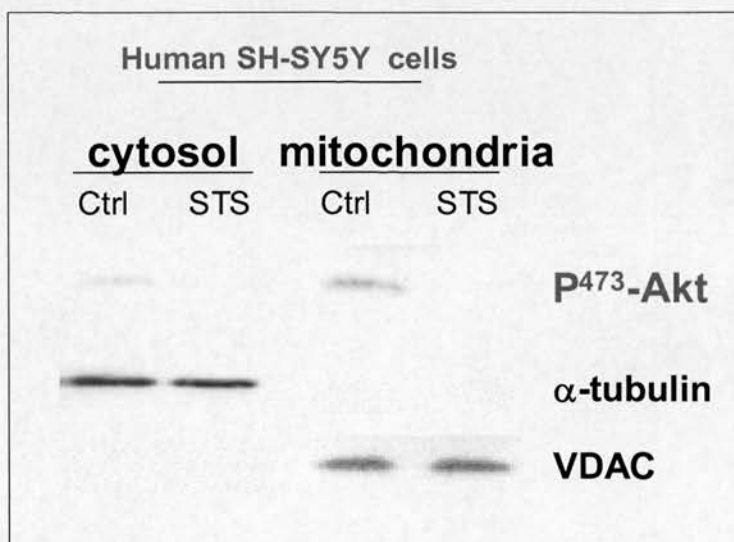


Figure 4.5: Loss of cytosolic and mitochondrial P⁴⁷³ Akt immunoreactivity in apoptotic SH SY5Y cells

Control and apoptotic (5 h/500nM STS) SH-SY5Y cells were isolated into cytosolic and mitochondrial fractions and assessed for changes in the subcellular distribution of endogenous P⁴⁷³ Akt (n=4). P⁴⁷³ Akt appeared both cytosolic and mitochondrial in origin, as well as, phosphorylated in untreated cells. In contrast, apoptosis was associated with dephosphorylation and a marked reduction both cytosolic and mitochondrial levels of phospho⁴⁷³ Akt. Equal loading and fraction purity was determined using antibodies against the cytosolic marker, α-tubulin and mitochondrial marker, VDAC.

Subcellular fractionation experiments were subsequently conducted to investigate whether Akt is also regulated by changes in subcellular distribution. Cytosolic and mitochondrial fractions of control and apoptotic (5 h/500nM STS) SH-SY5Y cells were analysed for changes in the subcellular distribution of P⁴⁷³ Akt, since this has been reported to be the active form of this protein. P⁴⁷³ Akt was detected both in the cytosol and at the mitochondria of untreated SH-SY5Y cells (*Figure 4.5*). In contrast, in apoptotic cells no P⁴⁷³ Akt could not be detected.

The nature of the interaction of P⁴⁷³ Akt at the mitochondrial membrane was subsequently investigated using alkali extraction methods, previously described for assessing the association of Bax and Bad at the outer mitochondrial membrane (OMM). Mitochondria from control and apoptotic SHSY-5Y cells were isolated into fractions containing proteins that were either ‘inserted’ or ‘attached’ into the OMM. P⁴⁷³ Akt immunoreactivity was detected in the ‘attached’, but not ‘inserted’ membrane fraction of control cells (*Figure 4.6*). STS-mediated apoptosis was associated with detachment of P⁴⁷³ Akt from the outer mitochondrial membrane. Alternatively, it might be argued that this might simply reflect the decreased expression levels of total P⁴⁷³ Akt, rather than Akt detachment from the OMM. Fraction purity was confirmed using Tom 20, an integral membrane protein.

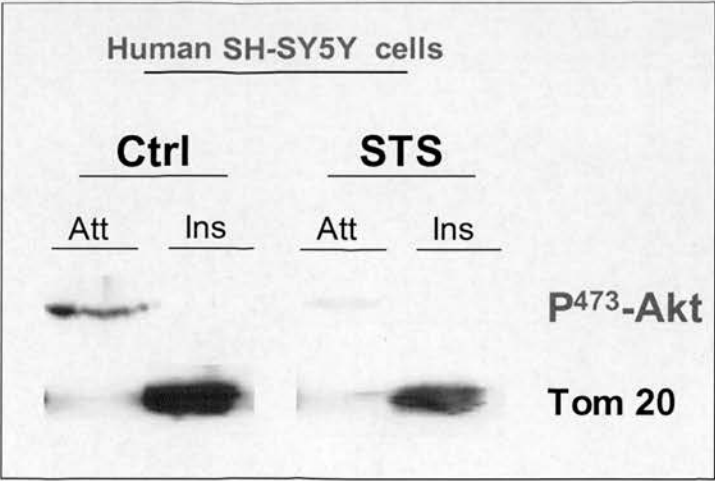


Figure 4.6: Apoptosis promotes detachment of P⁴⁷³ Akt from the OMM of SH-SY5Y cells.

Mitochondria of control and apoptotic (5 h/500nM STS) SH-SY5Y cells were isolated using an alkali extraction technique. P⁴⁷³ Akt appeared attached to the OMM of control SH-SY5Y cells. In contrast, apoptosis was associated with dephosphorylation and mitochondrial detachment of Akt. Tom 20, an integral membrane protein, confirmed fraction purity.

DISCUSSION

STS-mediated changes in both Bad and Akt were investigated to characterise the *in vitro* activation pathway for Bad in a model of apoptosis previously demonstrated to promote Bax translocation, with concomitant mitochondrial insertion (*see Chapter 3*). In contrast to Chapter 3, which reported upregulation of Bax protein expression in response to staurosporine treatment (STS; 5 h/500nM), no such changes were observed in Bad protein expression in this study. Serine residue (Ser) 112 and Ser-136 were identified as two major sites of phosphorylation and Bad inactivation (Zha *et al.* 1996). Ser-155, localised at the centre of the BH3 domain, was proposed critical in heterodimerization of Bad with anti-apoptotic members (Datta *et al.* 2000; Zhou *et al.* 2000). Datta *et al.* (2000) proposed that Ser-136 phosphorylation might precede Ser-155 phosphorylation by first binding 14-3-3 promoting a conformational change permitting access to Ser-155. However, Ser-155 phosphorylation has been reported independently of both Ser-136 and 14-3-3 (Tan *et al.* 2000, Zhou *et al.* 2000). Recent work supports the view that Ser-155 phosphorylation directly prevents heterodimerization by abolishing the affinity of the Bad BH3 domain for Bcl-X_L (Zhou *et al.* 2000). Both Ser-112 and Ser-136 lie within the potential 14-3-3 binding sites and may engage both binding sites on a 14-3-3 dimer (Zha *et al.* 1996). Initial studies to investigate the phosphorylation status of serine residue 112 failed due to difficulties in antibody detection and high background. Also, at the time of study, antibodies directed against serine-155 and serine-170 were not available. Therefore, this chapter investigated the phosphorylation status of serine residue 136, as a measure of Bad activity. Pro-apoptotic Bcl-2 family proteins have been reported to translocate towards the mitochondria upon receipt of an apoptotic stimulus (Adams *et al.* 2001, Makin *et al.* 2001). Subcellular fractionation experiments were therefore performed to as an alternative method of assessing protein activation. Using this technique, Bad activity appeared to be regulated by both phosphorylation status and subcellular distribution. Phosphorylation state was analysed using an antibody raised against phosphoserine (P)¹³⁶ Bad. Bad was observed in the cytosol of untreated SH-SY5Y cells, and appeared phosphorylated on serine residue 136. In contrast, apoptosis was associated with dephosphorylation of Bad (confirmed by the absence of changes in total protein levels) and concomitant mitochondrial accumulation of this pro-apoptotic protein. P¹³⁶ Bad was not observed in the mitochondrial fraction of either treatment group. These findings are in keeping with published findings (Zha *et al.* 1996, Datta *et al.* 1997).

Since serine residue 136 of Bad is one of several potential mediators in the PI3'K-Akt survival pathway (Datta 1999), and apoptosis-induced changes in P¹³⁶ Bad were observed in the present study, subsequent experiments were designed to investigate and correlate putative changes in Akt using the same *in vitro* model of apoptosis. Changes in total protein expression, phosphorylation status and subcellular distribution of this regulatory protein were investigated by Western blotting. These studies revealed lower levels of both total and P⁴⁷³ Akt immunoreactivity upon receipt of an apoptotic

stimulus. Subsequent subcellular fractionation experiments identified P⁴⁷³ Akt as a cytosolic and mitochondrial-associated protein in untreated cells. Interestingly, induction of apoptosis was associated with a marked reduction in all Akt and P⁴⁷³ Akt immunoreactivity. These findings are in keeping with the published data detailing loss of an Akt-mediated survival response following IL-3 withdrawal, with concomitant mitochondrial accumulation of dephosphorylated Bad (Datta *et al.* 1997). Furthermore, both PKA and Akt have been reported unable to phosphorylate Bad on residues Ser-112 and Ser-136, respectively, when Bad is in a pre-existing complex with Bcl-X_L (Strasser *et al.* 1997). Although substrate accessibility may be different in cells, this finding suggests that PKA and Akt may inactivate Bad, not by dissociating existing Bad/Bcl-X_L complexes at the mitochondria, but rather by preventing its accumulation in a dephosphorylated 'active' state. Consistent with this proposal, P¹³⁶ Bad in the present study was only detected in the cytosolic fraction of healthy cells. This supports the hypothesis that Akt might regulate pro-apoptotic Bad activity through preventative mechanisms in the cytosol, rather than displacement of putative Bad/Bcl-X_L complexes at the mitochondrial membrane.

Alkali extraction methods were subsequently used to characterise the association of P¹³⁶ Akt and pro-apoptotic Bad observed in the mitochondrial fraction of control and apoptotic SH-SY5Y cells, respectively. Apoptosis (5 h/500nM STS) was associated with translocation and mitochondrial accumulation of Bad, with concomitant attachment, but not insertion, of this protein to the outer mitochondrial membrane. These findings are consistent with the proposal that pro-apoptotic Bcl-2 family proteins devoid of a transmembrane (TM) domain are incapable of membrane insertion (Letai *et al.* 2002). Since a proportion of Bad remained cytosolic, failure to detect Bad insertion might be attributed to incomplete protein activation. However, this explanation seems unlikely, as this protein does not contain the necessary TM domain, proposed essential for membrane insertion (Letai *et al.* 2002). Separation of mitochondrial-associated proteins into those attached or inserted into the OMM revealed that P⁴⁷³ Akt was 'attached to', but not 'inserted into' the OMM of untreated SHSY-5Y cells. This is the first report of mitochondrial association for Akt, where STS-mediated apoptosis was associated with detachment of P⁴⁷³ Akt from the mitochondrial membrane. The implications of such findings await further investigation. Nevertheless, a hypothesised mechanism for Akt-mediated cell survival might be proposed based on findings from this study and in conjunction with other published data. Where inactivated and phosphorylated Bad could only be detected in the cytosol of healthy cells and mitochondrial accumulation of Bad was only detected in apoptotic cells, Akt might be hypothesised to have dual / independent functions within the cell, both at the cytosol and mitochondria. It is proposed that phosphorylated Akt, in turn, phosphorylates (and inactivates) Bad on serine-136 within the cytosol of healthy cells. In contrast, apoptosis results in dephosphorylation of Akt, both at the mitochondria and cytosol. In this scenario, dephosphorylated Akt might result in the accumulation of dephosphorylated Bad with concomitant mitochondrial localisation. In addition, mitochondrial associated phospho-Akt might regulate apoptotic cell death, possibly through binding

and thus activation of some pro-apoptotic Bcl-2 family protein target. In turn, dephosphorylation and mitochondrial detachment of Akt might permit mitochondrial accumulation of pro-apoptotic Bcl-2 family proteins, such as Bad.

Alternatively, Akt might serve independent functions within the cell. Cytosolic Akt might phosphorylate and thus inactivate pro-apoptotic Bad. Conversely, mitochondrial Akt might promote cell survival through regulation of other mitochondrial-associated proteins. Several possible mechanisms have been proposed. Data obtained using a motor neurone cell line, suggests that Akt is also capable of blocking apoptosis by acting at a step subsequent to cytochrome c release (Zhou *et al.* 2000). The identification of Akt substrates has been significantly aided by the characterisation of a consensus peptide motif (RXRXXpS/T) that is preferred by Akt (Alessi *et al.* 1996). Akt substrates that function downstream of cytochrome c release have not yet been defined, but may include components of the apoptosome and the IAPs. Human caspase-9 is a substrate of Akt (Cardone *et al.* 1998), but the importance of Akt-mediated caspase-9 phosphorylation as a general mechanism for cell survival is the subject of some controversy, as such an Akt phosphorylation site has not been detected in rodent caspase-9 (Fujita *et al.* 1999). Alternative post-mitochondrial targets for Akt might include APAF-1 and neuronal IAPs, both of which contain phosphorylation sites that are conserved across species. However, it remains to be determined whether APAF-1 or IAP are *bone fide* targets of Akt. An alternative mechanism of action for Akt at the mitochondria might be to potentiate calcium influx through L-type calcium channels (Blair *et al.* 1999), which may in turn promote neuronal survival through calcium-dependent pathways (Ghosh *et al.* 1995, Pap *et al.* 1998). It is worth noting that, because complete cytochrome c loss from the mitochondria is fatal even in the presence of survival factors, post-mitochondrial mechanisms used by Akt to promote survival likely represent fail-safe mechanisms against transient or low levels of cytochrome c release. However, the exact mechanism by which dephosphorylated Bad is directed towards the mitochondria and how this protein promotes cell death remains a subject of much debate.

Although the exact mechanism of action of Bad remains somewhat unclear, it has been argued whether Akt mediates Bad inactivation through altered expression of anti-apoptotic proteins, Bcl-2 or Bcl-X_L. For example, a previous study has shown that expression of active Akt decreases the levels of Bax protein induced by nitric oxide in primary hippocampal neurones (Matsuzaki *et al.* 1999). In contrast, Akt-mediated suppression of Bax translocation in STS-treated COS-1 cells was not due to increased protein expression of anti-apoptotic Bcl-2 family members (Tsuruta *et al.* 2002). Finally, in the present study, Akt detachment from the OMM correlated with the temporal profile of both Bax and Bad translocation, with mitochondrial insertion and attachment, respectively. However, characterisation of the temporal profile of both Bad and Akt redistribution would provide further insight into Akt regulatory mechanisms with control and apoptotic SHSY-5Y cells. To provide

evidence in support of a direct role for Akt in regulation of Bax activity would require the use of mutagenesis techniques. The rationale for studies performed within this thesis however, was to avoid such artificial conditions and to attempt to characterise the mechanism of action of pro-apoptotic Bcl-2 family proteins, under physiological conditions as would occur *in vivo*.

In conclusion, data reported in this chapter supports a model where Bad is phosphorylated on serine residue 136, and appears cytosolic in untreated SH-SY5Y cells. In contrast, apoptosis in this model was associated with dephosphorylation of Ser-136 with concomitant translocation of Bad towards the mitochondria. This was accompanied by attachment, but not insertion into the outer mitochondrial membrane. Also, the use of subcellular fractionation enabled detection of P⁴⁷³ Akt in both cytosolic and mitochondrial fractions of untreated cells. P⁴⁷³ Akt was attached, but not inserted into the OMM of control cells. In contrast, apoptosis in this model was associated with detachment of Akt from the mitochondria and a marked decrease in P⁴⁷³ Akt immunoreactivity in both organelle fractions. These findings are consistent with the proposal that BH3-only members devoid of a transmembrane domain cannot insert into the OMM and instead require binding to anti-apoptotic members to indirectly exert their death promoting activity. Therefore, Chapter 5 reports data from studies designed to characterise the mechanism of action of BNip3 and Nix, as examples of BH3-only proteins which contain a TM domain. These studies were designed to investigate whether Nix and/or BNip3 could also be activated in response to staurosporine treatment, and if so, whether apoptosis is accompanied by insertion of BNip3 or Nix into the OMM, consistent with the proposed structure / function relationship of the Bcl-2 protein family.

CHAPTER 5: *IN VITRO* CHARACTERISATION OF NIX

INTRODUCTION

Chapters 3 and 4 described the use of an STS-mediated model of apoptosis in human SH-SY5Y neuroblastoma cells used to characterise *in vitro* Bax and Bad activation, as examples of *multidomain* and *BH3 domain-only* (without a TM domain) pro-apoptotic Bcl-2 members, which have been implicated in the pathophysiology of experimental stroke. In contrast to all other known BH3-only members, Nix and its homologue BNip3, are unique in that they appear to be upregulated and activated in direct response to hypoxia, a finding that is of particular relevance with respect to characterising a pro-apoptotic Bcl-2 family response to focal cerebral ischaemia (*see Chapters 6 and 7*). Therefore, BNip3 and Nix activation were investigated as examples of BH3-only Bcl-2 family members, containing a TM domain.

BNip3 (Bcl-2 and Nineteen kDa Interacting protein-3) and Nix (Nip3-like protein X, also referred to as BNip3L), are members of a unique subfamily of BH3-only proteins. BNip3 was initially identified in yeast two-hybrid screening system, using adenovirus E1B 19K as bait (Boyd *et al.* 1994). Nix was originally assigned the name, BNip3-like protein (BNip3L), and considered a long version of BNip3 (Matsushima 1998). However, Chen *et al.* (1999) mapped this gene product to 14; 4.08, a distinct loci from the 8p21 chromosomal band assigned to *BNip3* gene, and suggested Nix should be considered a distinct protein (Imazu *et al.* 1998, Matsushima *et al.* 1998). These proteins share a high amino acid sequence homology, subcellular distribution and are subject to rapid proteosomal degradation (Cizeau *et al.* 2000, Ray *et al.* 2000). Human (h) and mouse (m) BNip3 share 56% and 53% sequence identity to hNix and mNix, respectively and 90.2% identity to each other. BNip3 and Nix localise to the endoplasmic reticulum, nuclear envelope and mitochondria (Boyd *et al.* 1994, Matsushima *et al.* 1998). When overexpressed, BNip3 (Chen *et al.* 1997) and Nix (Chen *et al.* 1999) co-localise with mitochondrial matrix protein, HSP 60. However, truncation of the critical TM domain results in a cytoplasmic and cytoplasmic/ primarily nuclear distribution of BNip3 and Nix, respectively (Chen *et al.* 1997, Chen *et al.* 1999).

The BH3 domain of BNip3 was initially reported essential for heterodimerization with both Bcl-X_L and E1B 19K and partially required for its pro-apoptotic activity (Yasuda *et al.* 1998). Although the BH3 domain of BNip3 contains only the minimal conserved amino acid residues, asparagine, leucine and isoleucine, Yasuda *et al.* (1998) demonstrated that substitution of this BH3 domain into the pro-apoptotic protein, Bax, functionally restored both heterodimerization and cell death activity in Bax. More recently, the TM domain of BNip3 and Nix, which share 80% sequence identity as well as the

amino terminus of these proteins, rather than the BH3 domain, was reported to be essential for mitochondrial localisation, heterodimerization and induction of cell death (Ray *et al.* 2000). Furthermore, the ability of truncated BNip3 and Nix to induce cell death suggested that each protein could, at least in part, promote cell death at extra-mitochondrial sites (Chen *et al.* 1999). Mtd/Bok (Inohara *et al.* 1998b, Hsu *et al.* 1997a) and Diva (Inohara *et al.* 1998a) have also been reported to promote cell death independent of their BH3 domain. An additional feature is that, unlike any other BH3only protein, BNip3 and Nix were reported to homodimerise (Boyd *et al.* 1994, Chen *et al.* 1999). BNip3 and Nix have been reported to contain a PEST sequence, suggesting rapid degradation and stage-specific expression (Boyd *et al.* 1994, Chen *et al.* 1997, Bruick 2000, Cizeau *et al.* 2000). Proteins are targeted to the proteasome via their PEST sequence for protein ubiquitination. This mechanism regulates apoptosis by promoting turnover of anti-apoptotic proteins and the degradation of potentially lethal pro-apoptotic proteins (Cizeau *et al.* 2000).

The ability to sense and respond to changes in oxygen levels is critical for many developmental, physiological and pathological processes, including angiogenesis, control of blood pressure, and cerebral and myocardial hypoxia. Hypoxia-Inducible Factor-1 α (HIF-1 α) is a basic helix-loop-helix (bHLH)-containing member of the PER-ANT-SIM (PAS) family of transcription factors that plays a central role in the cellular response to hypoxia (Carmeliet *et al.* 1998). Bruick (2000) demonstrated that embryonic stem cells where the gene encoding HIF-1 α has been disrupted, fail to undergo apoptosis under conditions of persistent hypoxia. Upon dimerisation with ARNT, HIF-1 α is stabilised and induces expression of target genes, which contain a functional HIF-1 α responsive element (HRE) in their transcriptional promoter. The BNip3 and Nix promoters were identified to contain canonical binding sites for HIF-1 α supporting the contention that these proteins might act as inducible factors to initiate cell death programs that are potentially activated by both hypoxia and forced expression of HIF-1 α (Bruick, 2000). HIF-1 α , and its relatives, are induced in response to hypoxia, and serve to coordinately activate the expression of target genes whose products act to facilitate cell survival (e.g. erythropoietin, VEGF, GLUT-1 and lactate dehydrogenase). However, following chronic hypoxia the protective responses fail and cells undergo apoptosis. Transcription of genes encoding BNip3 and Nix is greatly induced in response to hypoxia (Bruick, 2000, Vande Velde *et al.* 2000, Guo *et al.* 2001). This is in contrast to mRNA levels of other pro-apoptotic Bcl-2 members, such as Bax and Bad, which remained unaltered following periods of chronic hypoxia (Bruick, 2000).

Importantly, HIF-1 α is induced in the brain as a consequence of ischaemia, and appears to be required for delayed neuronal cell death (Halterman *et al.* 1999). Although significant advances in BNip3 and Nix research have been made in the last few years, most reported data was obtained using artificial conditions, such as site directed mutagenesis, over-expression, cell free systems or isolated mitochondria. This is of particular importance since over-expression was used in studies that reported

BNip3 and Nix to be novel mitochondrial-associated Bcl-2 family proteins (Chen *et al.* 1997, Chen *et al.* 1999). Such findings might not reflect the *in vivo* mechanism(s) of action of these proteins, but rather be an artifact of the experimental technique used. A potentially critical finding was that these two homologues appear to promote cell death, in response to a given stimulus, through distinct mechanisms of action. Nix was observed to promote cytochrome c-dependent apoptosis (Imazu *et al.* 1999), similar to other pro-apoptotic Bcl-2 family proteins (Green & Reed 1998). In contrast, BNip3 was reported to activate a novel form of cell death resembling necrosis (Vande Velde *et al.* 2000). It was therefore anticipated that these two hypoxia-sensitive Bcl-2 family proteins might have specific roles to play in transduction and integration of cell death signals following conditions of hypoxia, specifically during focal cerebral ischaemia and considered necessary to further characterise the putative mechanisms of action of BNip3 and Nix using different *in vitro* models of apoptosis.

AIMS

The aims of this chapter were to: (a) provide a positive control for Nix and BNip3 protein expression for future *in vivo* studies using Western blotting, (b) compare and contrast the temporal profile of hypoxia-induced Nix activation with previously reported BNip3, (c) compare and contrast the observed mechanisms of action of both pro-apoptotic BNip3 and its homologue Nix with available published data and finally (d) investigate whether these proteins could be activated *in vitro* by stimuli, other than hypoxia.

MATERIALS & METHODS

Subcellular fractionation and Western blotting (*see Chapter 3*) were used to investigate apoptosis-induced changes in BNip3 and Nix protein expression. However, experimental conditions specific to this chapter will be described below.

Induction of apoptosis in Chinese Hamster Ovary cells

Chinese Hamster Ovary (CHO-K1) cells were cultured in HAM-F12 growth medium containing, 10% FCS, 2mM L-Glutamine, 100 units/ml Penicillin and 100mg/ml Streptomycin. Cells were grown to confluency and transferred to a humidified hypoxic chamber. It has been reported difficult to induce cell death in CHO-K1 cells in the presence of normal serum conditions (Bruick 2000). Therefore,

serum levels were reduced to 0.5% FCS on day 1 of the experiment. Cells were exposed to 1-7 days @ 1% O₂ / 0.5% FCS and lysed using the standard detergent-free SCF extraction buffer (see Chapter 3). Apoptosis was confirmed by increased immunoreactivity to cleaved caspase-3 fragments in hypoxic compared to control cells using standard Western blotting techniques.

Analysis of Protein Expression by Subcellular Fractionation and Western blotting

Human SH-SY5Y and mouse A1.1 cells were grown to confluency and apoptosis was induced as previously described (*see Chapter 3*). Control and apoptotic CHO-K1 cells (4 days hypoxia), SH-SY5Y (5 h/500nM STS) and A1.1 cells (18 h/200nM DEX) were lysed and cytosolic and mitochondrial fractions were extracted by subcellular fractionation (*see Chapter 3*). Proteins were subsequently separated by SDS PAGE gel electrophoresis and analysed by Western blotting. Primary antibodies used were: anti-BNip3L (1:1000; ψ ProSci Inc.), anti-cleaved capase-3 (1:1000; New England BioLabs), anti-BNip3 (1:2500; Santa Cruz) or BNip3 antiserum (*gift from Richard Bruick, University of Texas, USA*). Membranes were also incubated with antibodies raised against β -tubulin (1:1000), α -tubulin (1:2000) and VDAC (1:2000; all Sigma) to ensure equal loading, protein transfer and fraction purity in total cell lysates, cytosol and mitochondrial fractions, respectively. The immunoreactive proteins were visualized using the appropriate horseradish peroxidase-coupled anti-rabbit or anti-mouse Ig secondary antibody (1:2500; Amersham Life Sciences), or anti-goat Ig secondary antibody (1:5000; Sigma) and enhanced chemiluminescence (ECL; Amersham Life Sciences).

RESULTS

Total cell lysates from control and hypoxic (1-7 day @ 1% O₂/5% FCS) CHO-K1 cells were analysed for changes in cleaved caspase-3 protein expression levels. A progressive increase in caspase-3 cleavage products (19 kDa) was detected following 2-7 days hypoxia (*Figure 5.1*). Cell lysates from the treatment groups were analysed for changes BNip3 and Nix protein expression using antibodies directed against either Nix (anti-BNip3L) or BNip3. BNip3 could not be detected using either BNip3 antibody (data not shown), although high background and non-specific binding were observed with each of these antibodies. Since the original study by Bruick and colleagues (2000) reported a paralleled increase in Glut-1 and BNip3 protein expression in hypoxic CHO-K1 cells, putative changes in Glut-1 protein expression were therefore investigated. In keeping with the studies of

Bruick, a progressive increase in Glut-1 expression was observed following 3-7 days hypoxia (Figure 5.2).

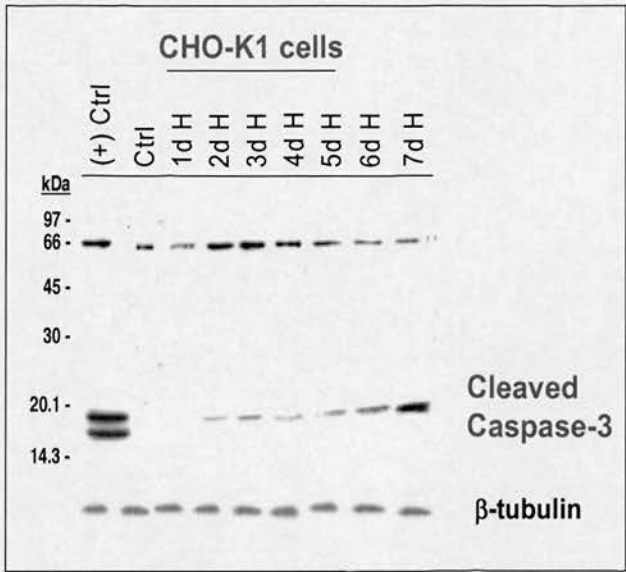


Figure 5.1: Confirmation of apoptosis in hypoxic CHO-K1 cells

Total cell lysates (50 μ g) from control and hypoxic (1-7 day @ 1% O₂/5% FCS) CHO-K1 cells were analysed for changes in cleaved caspase-3 protein (n=5). A progressive apoptotic response, measured by an increase in caspase-3 cleavage products, was detected following 2-7 days hypoxia. Caspase-3 expression in DEX-treated A1.1 cells confirmed apoptosis (cleaved fragments; 17 kDa and 19 kDa). β -tubulin blotting confirmed equal loading.

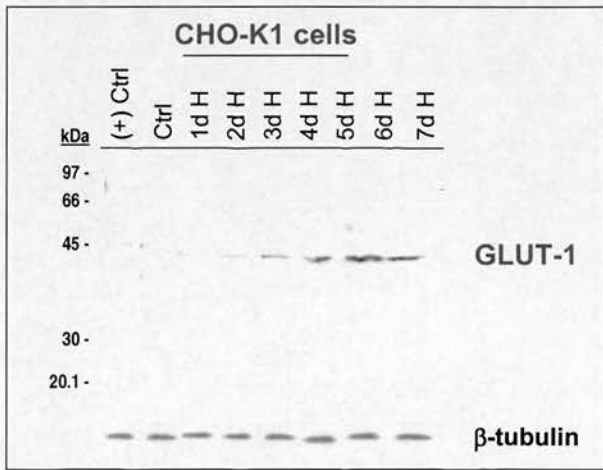


Figure 5.2: Hypoxia-mediated changes in Glut-1 protein expression in CHO-K1 cells

Cell lysates from control and hypoxic (1-7 day @ 1% O₂/5% FCS) CHO-K1 cells were analysed for changes in Glut-1 protein expression (n=5). A progressive increase in Glut-1 protein expression was observed between 2-7 days hypoxia.

Two immunoreactive bands were identified using an antibody raised against Nix in experimental samples and in K562 cell lysates, supplied with the Nix antibody as a positive control. These two immunoreactive proteins migrated on SDS PAGE gels with apparent molecular weights corresponding to Nix (48 kDa) and 30 kDa protein. *In vivo* transcription and translation of [³⁵S] methionine-labelled BNip3 identified 30kDa and 60kDa alkali- and urea-resistant bands corresponding to a monomer and dimer of the BNip3 protein (Chen *et al.* 1997). High sequence identity between Nix and BNip3 suggests that the 30 kDa immunoreactive protein identified might be a monomer of BNip3. In the absence of any definitive proof, however, this protein will be referred to as a Nix-like protein. A progressive increase in the 30 kDa protein expression levels was observed following 4-7 days hypoxia in while cell lysates (Figure 5.3). However, this protein response was observed after onset of apoptosis; where cleaved caspase-3 immunoreactivity was detected after 2 days hypoxia (Figure 5.1). In contrast, Nix could not be detected, or was below levels of detection, in this experiment.

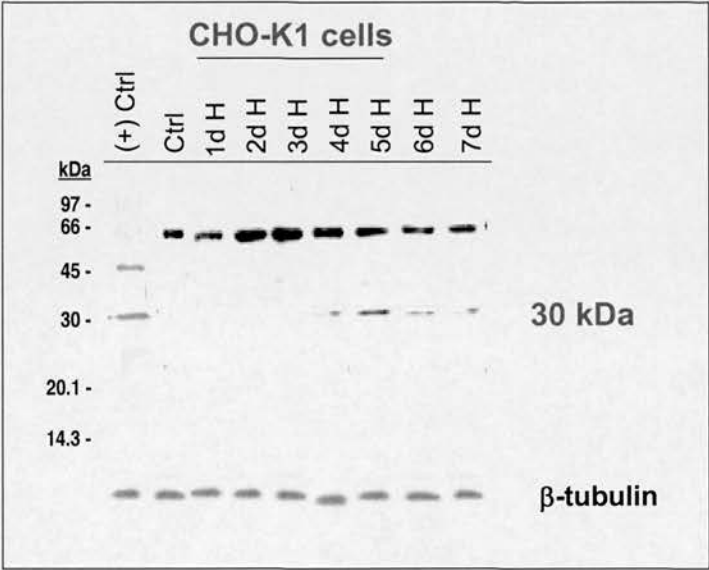


Figure 5.3: Hypoxia-induced changes in Nix protein expression

Cell lysates from control and hypoxic (1-7 day @ 1% O₂/5% FCS) CHO-K1 cells were analysed for changes Nix protein expression (n=5). Two immunoreactive bands, which migrated as 48 kDa and 30 kDa proteins were identified in K562 cells (positive control). A progressive increase in expression of the 30 kDa Nix-like protein (possibly a monomer of BNip3) was detected between 4-7 days hypoxia. In contrast, Nix was not detected at any time point. β-tubulin confirmed fraction purity.

Data presented in the previous chapters demonstrated Bax (Chapter 3) and Bad (Chapter 4) translocation towards the mitochondria upon receipt of an apoptotic stimulus. Subcellular fractionation was performed in control and apoptotic (4 days hypoxia) CHO-K1 cells to investigate whether the Nix-like protein were also subject to similar post-translational modifications. The

hypothesis that Nix was expressed in low levels in this cell type and might only be detected in enriched cell fractions was also tested. Nix (48 kDa) was detected at low levels in the cytosol of untreated CHO-K1 cells. In contrast, the 30 kDa Nix-like protein could not be detected in either fraction, or was below levels of detection (*Figure 5.4*). In contrast, apoptosis was associated with upregulation of cytosolic Nix and *de novo* synthesis of the 30 kDa Nix-like protein in the mitochondrial fraction. Translocation of Nix towards the mitochondria was not observed.

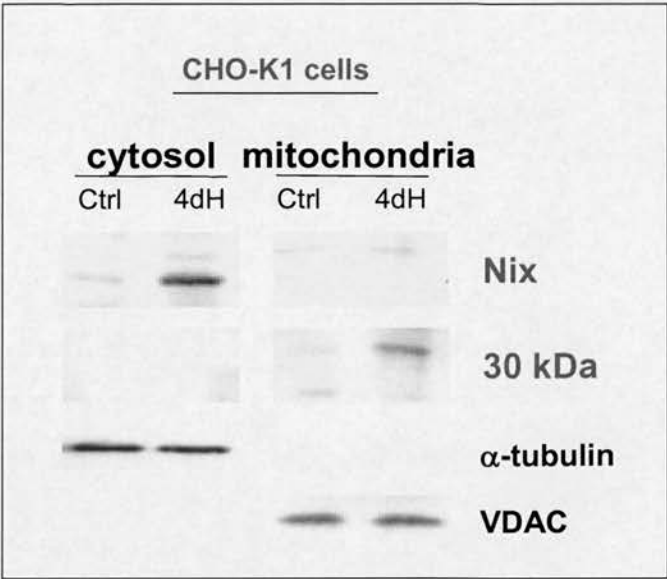


Figure 5.4: Hypoxia-induced changes in Nix subcellular localisation

Enriched cytosolic and mitochondrial fractions from control and hypoxic (4 days @ 1% O₂/5% FCS; 4dH) CHO-K1 cells were assessed for changes in the subcellular distribution of Nix (48 kDa) and the Nix-like (30 kDa) protein (n=4). Nix was detected at low levels in the cytosol of untreated cells. The 30 kDa Nix-like protein could not be detected in either fraction. In contrast, hypoxia was associated with upregulation of Nix and the Nix-like protein, in the cytosol and mitochondria, respectively. Translocation of either protein was not observed. VDAC and α-tubulin confirmed fraction purity and equal loading in the mitochondrial and cytosolic fractions, respectively.

Subsequent studies were performed using two other *in vitro* models of apoptosis: staurosporine-treated human SH-SY5Y neuroblastoma cells (5 h/500nM STS) and dexamethasone-treated mouse A1.1 monocytes (18 h/200nM DEX) (*see Chapter 3*). The aim of this experiment was to investigate whether or not pro-apoptotic Nix (and/or the Nix-like protein) could be activated by stimuli, other than over-expression (Chen *et al.* 1997, Chen *et al.* 1999) and hypoxia. Total cell lysates from control and apoptotic SH-SY5Y and A1.1 cells were analysed for changes in Nix and Nix-like protein expression (*Figure 5.5*). Endogenous Nix (48 kDa) was detected at low levels in each cell type. Apoptosis induced with staurosporine (SH-SY5Y cells) or dexamethasone (A1.1 cells) was associated with a marked upregulation of Nix (48 kDa) protein expression in each cell type. In contrast, the 30 kDa protein was not detected in the total cell lysate of either cell type, even after induction of apoptosis.

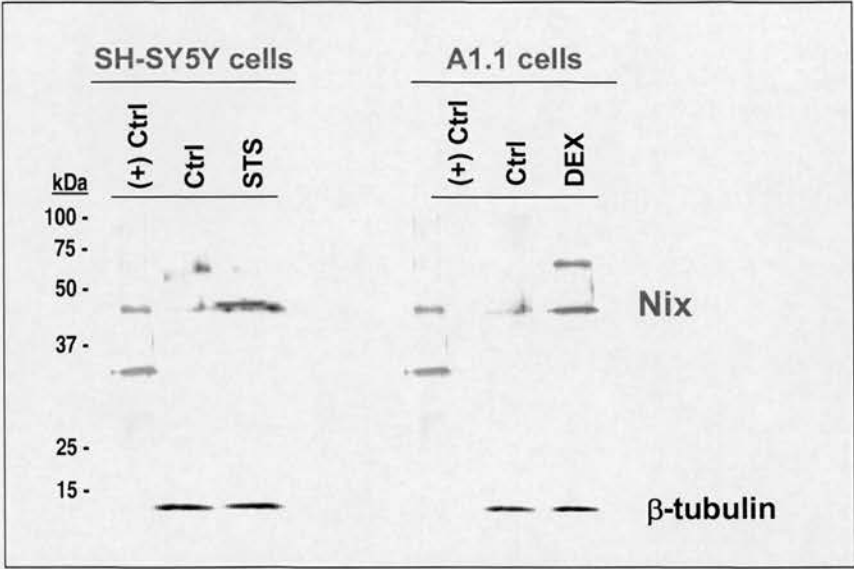


Figure 5.5: STS- and DEX-mediated changes in Nix protein expression levels

Lysates from control and apoptotic SH-SY5Y (5 h/500nM STS) and A1.1 cells (18 h/200nM DEX) were assessed for changes in Nix and Nix-like protein expression (n=4). Upregulation of Nix (48 kDa) was observed in the apoptotic compared to untreated group of each cell type. In contrast, a 30 kDa immunoreactive band was not detected in any treatment group. K562 cell lysates provided the positive control. Equal loading was confirmed using β-tubulin.

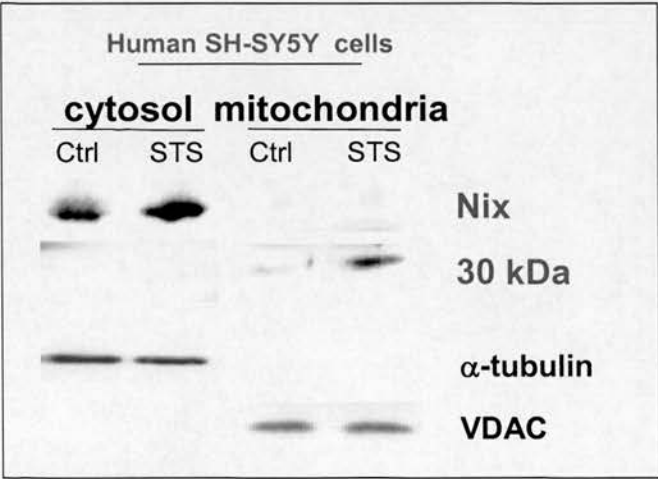


Figure 5.6: STS-mediated changes in the subcellular distribution of pro-apoptotic Nix

Cytosolic and mitochondrial fractions were obtained from control and apoptotic (5 h/500nM STS) SH-SY5Y cells and assessed for changes in the subcellular distribution of Nix (n=4). Apoptosis was associated with a marked increase in cytosolic levels of Nix (48 kDa). Upregulation of a second immunoreactive protein (30 kDa) was observed in the mitochondrial fraction upon receipt of an apoptotic stimulus. Equal loading and fraction purity were confirmed by cytosolic and mitochondrial protein markers, α-tubulin and VDAC, respectively.

Subcellular fractionation experiments were subsequently performed to further characterise putative pro-apoptotic Nix and Nix-like proteins responses to staurosporine- and dexamethasone-mediated apoptosis. Enriched cytosolic and mitochondrial fractions of control and apoptotic SH-SY5Y cells were prepared and analysed by Western blotting. Similar to findings in CHO-K1 cells, Nix was observed primarily as a cytosolic protein in untreated SH-SY5Y cells (*Figure 5.6*) and apoptosis was associated with upregulation of cytosolic Nix. Mitochondrial levels of Nix were very low and did not change in response to apoptosis. Again, the 30 kDa Nix-like protein was observed only in the mitochondrial fraction, and was upregulated in response to an apoptotic stimulus.

DISCUSSION

Existence of EGL-1, a BH3 only ortholog in the worm, supports a critical role for this sub-group of Bcl-2 family proteins in apoptotic cell death (Conradt *et al.* 1998, del Peso *et al.* 1998). Interestingly, unlike any other mammalian members identified to date, BNip3, and its homologue Nix, have a specific *C. elegans* counterpart, termed ceBNip3 (Cizeau *et al.* 2000), suggesting that these proteins might have a distinct, but as yet unidentified, role within the cell. In a landmark study, Bruick (2000) demonstrated that the mRNA of BNip3 and Nix was markedly elevated in direct response to hypoxia. This elevation was not observed for other pro-apoptotic Bcl-2 family members investigated. On the basis of these observations, Bruick suggested that BNip3 and Nix should be considered novel hypoxia-responsive Bcl-2 family members that might be of particular importance during conditions of persistent hypoxia, such focal cerebral ischaemia. Importantly, most studies investigating these two proteins have employed artificial conditions (Chen *et al.* 1997, Matsushima *et al.* 1998, Chen *et al.* 1999). For example, Chen and colleagues characterised both BNip3 (Chen *et al.* 1997) and Nix (Chen *et al.* 1999) as unique mitochondrial Bcl-2 family proteins based on findings from transiently transfected Rat-1 and MCF-7 human carcinoma cells in which both BNip3 and Nix were over-expressed. It was therefore decided that the *in vitro* activation pathway of these two endogenous proteins should be investigated, with the aim of subsequently comparing such findings with the observed *in vivo* mechanism(s) of action (*see Chapters 6 and 7*).

Initial attempts were made to reproduce the findings of Bruick (2000) and to provide a positive control for both BNip3 and Nix for use in further studies. An apoptotic model of chronic hypoxia (with serum deprivation) was set-up in Chinese hamster ovary (CHO-K1) cells, as described by Bruick (2000). Unfortunately, BNip3 could not be detected in this system - using either BNip3 antiserum (gift from Richard Bruick) or a commercially available antibody which became available during these studies - due to high background and to lack of antibody specificity. However, induction of apoptosis was confirmed by the detection of caspase-3 cleavage fragments following 2-7 days hypoxia compared to

control. In addition, a progressive upregulation of Glut-1 protein expression (a well characterised hypoxia-responsive gene dependent upon HIF-1 α activation) was observed between 4-7 days hypoxia (Bruick, 2000). These findings are comparable with the observed Glut-1 expression pattern in Bruick's study, although caspase-3 expression was not investigated (Bruick, 2000). It was therefore anticipated that lack of an observed BNip3 response was likely a result of antibody problems, rather than a lack of BNip3 protein response *per se*. The subsequent availability of a new Nix antibody permitted the characterization of two specific immunoreactive proteins, which migrated as 48 kDa and 30 kDa proteins under SDS-reducing conditions. Antibody specificity was confirmed using K562 cell lysates as a positive control). Unlike the predicted 23.8kDa molecular weight for Nix, this protein runs as a 48kDa dimer under reducing conditions using SDS gel electrophoresis (Chen *et al.* 1999). Unfortunately, since attempts to characterize this 30 kDa protein using 2D gel and mass spectrometry (Maldi-Tof) failed (*performed by Dr. Duncan Short, data not shown*), it was not possible to ascertain whether or not this protein was in fact a monomer of BNip3. However, the high homology between both Nix and BNip3 would suggest that it is likely that the Nix antibody might react with both Nix and BNip3 proteins (Figure 5.7, Chen *et al.* 1999).

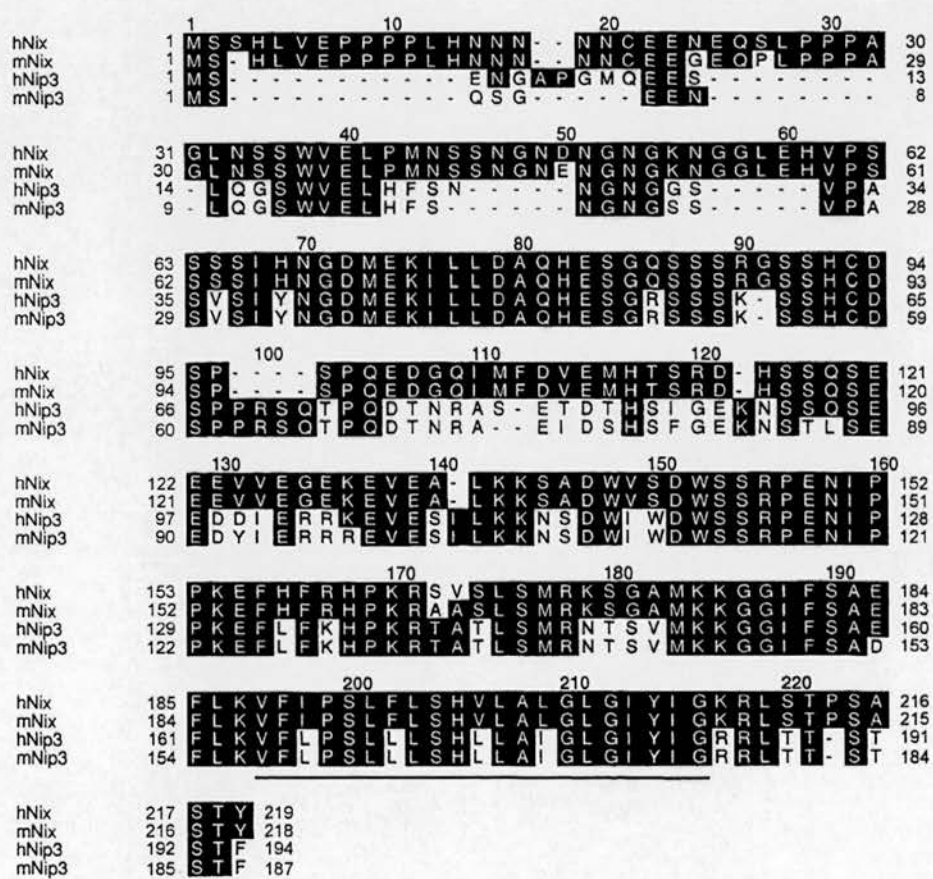


Figure 5.7: Alignment of predicted murine and human Bnip3 and Nix proteins. Regions of identity with human Nix are shaded. The predicted transmembrane domains are underlined (from Chen *et al.* 1999).

In addition, *in vivo* transcription and translation of [³⁵S] methionine-labelled BNip3 identified 30kDa and 60kDa alkali- and urea-resistant bands corresponding to a monomer and dimer of the BNip3 protein. This is in contrast to 21.54kDa predicted from the amino acid sequence (Chen *et al.* 1997). Although it was hypothesised that the second immunoreactive protein represented a monomer of BNip3, from herein it will be referred to as the 'Nix-like' protein. Interestingly, in keeping with findings from Bruick's study (2000), upregulation of Nix and the Nix-like protein were observed following 4-7 days hypoxia. Interestingly, caspase-3 cleavage appeared to precede both Nix and the Nix-like protein activation. This might reflect activation of other pro-apoptotic Bcl-2 family proteins and/or other apoptotic mediators in this model of apoptosis. For example, Bax translocation and cytochrome c release have been reported in many cell types exposed to hypoxia (Saikumar *et al.* 1998).

Subcellular fractionation techniques were used to characterise possible changes in the subcellular distribution of both Nix and the 30 kDa Nix-like protein in control and hypoxic CHO-K1 cells. In contrast to previous reports which used transient transfection techniques (Chen *et al.* 1997, Chen *et al.* 1999) and subsequently suggested BNip3 and Nix to be mitochondrial proteins, the present study identified Nix as a primarily cytosolic protein in untreated CHO-K1 cells. Conversely, the Nix-like protein could only be detected in the enriched mitochondrial fraction. Hypoxia was associated with *de novo* synthesis / upregulation of both immunoreactive proteins. Unfortunately, attempts to investigate the nature of the Nix-like protein's association at the mitochondria of control and hypoxic CHO-K1 cells proved unsuccessful (data not shown). It is anticipated that the advent of more specific antibodies or with higher affinities may facilitate characterisation of the association of the Nix-like immunoreactive protein at the mitochondria. Mitochondrial accumulation of Nix was not detected; however it is possible that the hypoxic stimulus used was insufficient to induce translocation of cytosolic Nix. Findings from the present study suggested that, at least in CHO-K1 cells, Nix was expressed at higher levels than the 30 kDa, Nix-like protein. However, this might merely reflect the different affinities of the Nix antibody for these two proteins. Assuming that the 48 kDa protein represents a Nix dimer, then this is the first study to report Nix as an endogenously expressed cytosolic protein. However, identification of both the 30 kDa and 48 kDa proteins await confirmation. Supporting this proposal, Nix was identified as being a distinct Bcl-2 family member, with a potentially distinct mechanism of action (Chen *et al.* 1999), rather than being simply an elongated form of BNip3 (Matsushima *et al.* 1998, Imazu *et al.* 1998).

In a subsequent study, two *in vitro* models of apoptosis were used to investigate whether or not Nix could be activated by stimuli, other than hypoxia (Bruick *et al.* 2000, Guo *et al.* 2001, Regula *et al.* 2002). Chapters 3 and 4 identified that Bax and Bad were activated following staurosporine treatment of human SH-SY5Y neuroblastoma cells and dexamethasone treatment of mouse A1.1 monocytes. In

whole cell lysates, Nix, but not the 30 kDa Nix-like protein, was observed in each cell type investigated. STS- and DEX-mediated apoptosis was associated with upregulation of Nix, but not the Nix-like protein expression. Data obtained in Chapters 3 and -4 confirmed that Bax and Bad are both regulated by post-translational modifications. Mitochondrial accumulation of these proteins appeared a common event following an apoptotic stimulus. More importantly, since pro-apoptotic Bad appeared to be regulated by changes in subcellular distribution, rather than protein synthesis *per se*, the present study also used subcellular fractionation techniques to further investigate putative Nix and Nix-like protein responses to hypoxia. Cytosolic and mitochondrial fractions of control and STS-treated SH-SY5Y cells were used to further characterise these two proteins. In agreement with data obtained using CHO-K1 cells, Nix and the 30 kDa protein were observed in the enriched cytosolic and mitochondrial fraction, respectively, in untreated SH-SY5Y cells. STS-mediated apoptosis was associated with upregulation of both cytosolic Nix and the 30 kDa mitochondrial protein. Again, as observed in hypoxic CHO-K1 cells, Nix translocation could not be detected in STS-treated SH-SY5Y cells, or was below present limits of detection. In untreated SH-SY5Y cells, Nix also appeared to be expressed at higher levels than the 30 kDa protein, although again, this might reflect antibody specificity, rather than protein levels, *per se*. As suggested above in respect of hypoxic CHO-K1 cells, it is possible that lack of Nix translocation/mitochondrial accumulation in these models may be due to an insufficient apoptotic stimulus. Whether a more severe insult might promote Nix translocation, remains to be investigated. Alternatively, over-expression and artificial conditions might be responsible for inducing mitochondrial localization of Nix, as demonstrated in other studies (Matsushima *et al.* 1998, Imazu *et al.* 1998). However, the spatial profile of the 30 kDa protein in both control and apoptotic CHO-K1 and SH-SY5Y cells is similar to previously reported findings for BNip3, providing further support for this band representing a BNip3 monomer. Attempts to characterise the type of interaction of this specific immunoreactive protein with the mitochondrial membrane proved unsuccessful, and warrant further investigation. This was the first study to characterise the mechanisms of action of Nix, and the immunoreactive 30 kDa Nix-like protein following three disparate apoptotic stimuli. These studies identified a novel *in vitro* activation pathway for Nix, which appeared common to all three different stimuli and cell types. While previous studies reported a mitochondrial distribution for Nix, mostly through the use of over-expression studies (Chen *et al.* 1999), the novel finding of endogenously expressed cytosolic Nix with upregulation but not translocation in the present study, suggests cytosolic upregulation might be an early event in the Nix activation pathway (*see Chapter 3*). Nonetheless, these findings reinforce the necessity to characterise the activation pathways for these proteins in the absence of artificial conditions, which may not reflect the *in vivo* activation pathway.

Nix is reported to promote cytochrome c-dependent apoptotic cell death in isolated mitochondria (Imazu *et al.* 1999, Vande Velde *et al.* 2000). BNip3, in contrast, appears to activate a novel form of cell death that resembles both morphological and biochemical features of necrosis involving PTP

opening and mitochondrial membrane depolarization (Cizeau *et al.* 2000, Vande Velde *et al.* 2000, Regula *et al.* 2002). BNip3-mediated cell death has been reported to progress in the absence of cytochrome c release, caspase-3 and caspase-9 activation (as demonstrated using pan-caspase inhibitor Ac-zVAD-fmk) (Vande Velde *et al.* 2000). Moreover, cell death in this system was associated with morphological characteristics of plasma membrane permeability changes, cytoplasmic vacuolation, mitochondrial rounding and autophagy with a morphotype of necrosis, but without mitochondrial swelling, resembling cell death observed in Fas-treated Jurkat cells (Vande Velde *et al.* 2000). Furthermore, DNA fragmentation and chromatin condensation appeared as a late event, likely once the cells were already committed to death. This event was only partially inhibited by Ac-zVAD-fmk, and independent of AIF translocation (Vande Velde *et al.* 2000, Regula *et al.* 2002). Forced expression of BNip3 in transiently transfected 293T cells resulted in nuclei with mottled, dispersed foci of chromatin condensation, rather than global condensation as observed following caspase-mediated cell death (Vande Velde *et al.* 2000). However, the relevance of such findings with respect to the apparent mechanism of action of BNip3 is unclear. For example, both Bax and Bak-mediated cell death have been reported to progress, albeit without nuclear changes, in the presence of the pan-caspase inhibitor, Ac-zVAD-fmk (Vande Velde *et al.* 2000).

Nix and the 30 kDa Nix-like protein activation were observed in the staurosporine model of apoptosis previously reported to involve cytochrome c release as well as caspase-3 and caspase-9 cleavage (*see Chapter 4*). It is possible however, that activation of alternative proteins, such as Bax or Bad, mediated cytochrome c release and caspase activation. Recent findings from a study using adult rat ventricular myocytes from animals suffering chronic heart failure and exposed to hypoxic conditions, observed BNip3 upregulation and insertion into the OMM prior to cell death (Regula *et al.* 2002). Moreover, in contrast to findings using over-expression systems, hypoxic-mediated cell death of cardiomyocytes was associated with both caspase activation and cytochrome c release (Regula *et al.* 2002). These findings are similar to observations in the STS model of apoptosis described in this thesis (*see Chapter 4*). One possible explanation might be that following either hypoxia or STS, a cacophony of pro-apoptotic mechanisms might be activated simultaneously whereby BNip3-induced $\Delta\psi_m$ loss and PTP changes might comprise just one response. Cytochrome c release and caspase activation might be mediated through activation of other Bcl-2 family members, such as Bax, Bad or indeed Nix. In support of such a proposal is the finding that Bax is activated in both aforementioned apoptotic models (Jurgensmeier & Reed 1998, McGinnis *et al.* 1999). In this scenario, it might be proposed that in hypoxic or STS-treated cells endogenous Nix and BNip3 are simultaneously activated and act 'in synchrony' to promote the previously observed manifestations of cell death in these systems.

Although increased availability of antibodies to both BNip3 and Nix will no doubt dramatically facilitate characterization of these two cell death pathways, current knowledge of the BNip3- and Nix-mediated molecular cell death events remains incompletely understood, and somewhat confusing. The mechanism by which Nix, BNip3 and/or the 30 kDa immunoreactive protein, promote cytochrome c release and/or PTP-like changes in STS-treated SH-SY5Y cells and other apoptotic cell systems is a matter of much controversy, and warrants further investigation. Clearly, findings from the present study suggest Nix, and possibly BNip3 or another as yet unidentified Nix-like 30 kDa immunoreactive protein, can be activated by stimuli other than hypoxia. Whether Nix promotes cytochrome c release through heterodimerization with Bax, or other family members, also warrants further investigation. The identification of a functionally similar *C. elegans* ortholog, ceBNip3, suggests that this specific subgroup of evolutionary conserved pro-apoptotic Bcl-2 family proteins might play a specific role(s) in the cell that cannot be substituted by other pro-apoptotic family members. One such role might lie within their direct response to hypoxia. Since morphological features of both necrosis and apoptosis have been reported within the same brain regions following focal cerebral ischaemia (Linnik *et al.* 1994), tackling the issue of whether Nix, BNip3 and/or the Nix-like proteins are activated simultaneously, will no doubt facilitate our understanding of the cell death mechanisms during conditions of hypoxia.

In vitro characterisation of Bax, Bad and Nix identified that each protein could be activated in response to a given apoptotic stimulus. Data obtained in Chapters 3, -4 and -5 suggested that, in addition to protein-specific responses, mitochondrial accumulation was likely a critical event in transduction of each protein's pro-apoptotic response. Using subcellular fractionation as a more accurate measure of protein activity, subsequent studies were designed to investigate putative Bax, Bad and Nix responses to focal cerebral ischaemia. Initial stroke studies (*see Chapter 2*) failed to detect apoptotic changes, therefore models of focal cerebral ischaemia involving varied occlusion and reperfusion durations were set-up in attempts to characterise pro-apoptotic changes under less severe conditions of ischaemia.

CHAPTER 6: PERMANENT FOCAL ISCHAEMIA

INTRODUCTION

Chapter 2 described the setting up and validation of rat MCA occlusion models. Although a well demarcated cortical and striatal lesion was observed following 24 h transient (2 h) ischaemia, consistent with laboratory and literature standards, no evidence was obtained to support a role for apoptotic cell death in this ischaemic stroke model. However, these findings are in contrast to studies which have reported DNA fragmentation between 6 h and 24 h ischaemia (De Girolami *et al.* 1984, Li *et al.* 1995, Gillardon *et al.* 1996, van Lookeren Campagne *et al.* 1996, Krupinski *et al.* 2000). More recently, several independent studies have reported a Bcl-2 family response during focal cerebral ischaemia (Gillardon *et al.* 1996, Isenmann *et al.* 1998, Kitagawa *et al.* 1998, Prakasa *et al.* 2000), further supporting a specific role for this family in the pathophysiology of stroke.

As discussed in Chapter 2, the reasons for the lack of an observed apoptotic response remain unclear, however, severity of the ischaemic insult and inappropriate methods of assessing Bcl-2 family activity are two possible explanations. Whether a cell dies by apoptosis or necrosis has been proposed to be dependent upon the severity and duration of the ischaemic insult (Linnik *et al.* 1993, MacManus *et al.* 1997), therefore it was hypothesised that altering the ischaemic duration might 'unveil' the apoptotic response (Choi *et al.* 1996). Subcellular fractionation and confocal microscopy techniques were established *in vitro*. Chapters 3, -4 and -5 characterised the mechanism of action of Bax, Bad and Nix *in vitro* and confirmed the use of these techniques to be a more sensitive measure of Bcl-2 family protein activity. As such, mitochondrial accumulation was considered to be a critical event in the signal transduction pathway of this family of regulatory proteins.

Although, DNA fragmentation and mRNA expression levels are commonly used to assess apoptosis (Gillardon *et al.* 1996, Davoli *et al.* 2002, Hu *et al.* 2002), the relevance of such findings has been questioned. Investigating changes in the subcellular distribution of Bax, Bad and Nix, together with cleaved caspase-3 immunoreactivity and morphological assessment might be a more appropriate method of assessing Bcl-2 family protein activation and the role of apoptotic cell death in focal cerebral ischaemia. Therefore, by developing *in vitro* protocols established in Chapter 3, this chapter investigated the effects of increased ischaemic duration on pro-apoptotic Bcl-2 family protein activity in a rat model of permanent focal cerebral ischaemia.

AIMS

The aims of this chapter were to (a) investigate the effects of ischaemic duration on histological and biochemical features of cell death, after 6 h and 24 h permanent MCA occlusion, (b) compare and contrast putative pro-apoptotic Bcl-2 family response(s) with *in vitro* data obtained in Chapters 3, -4 and -5.

MATERIALS & METHODS

The materials and methods for the histological and biochemical data presented in this chapter were described comprehensively in *Chapters 2 and -3*.

Histological Assessment of Damage

Briefly, male Sprague Dawley rats (275-315g, aged 8-11 weeks) were subjected to permanent (PMF) monofilament occlusion of the right MCA under halothane/N₂O anaesthesia (*see Chapter 2*). The necks of sham-operated animals were incised, with insertion and immediate removal of the monofilament. Animals were allowed to recover for 6 h or 24 h. Following transcardiac perfusion, under deep anaesthesia, brains were removed and post-fixed in 20% sucrose in PFA/PBS solution. Thionin staining was used to identify infarct margins and sections were subsequently re-stained using haematoxylin and eosin (H&E) to further characterise nuclear changes associated with ischaemic cell death (*see Appendix A*). Brain damage was quantified using a microcomputer imaging device (MCID) and volume calculated according to the trapezoid rule. Volume of damage (mm³) was expressed as the mean \pm standard error of the mean (s.e.m.).

Biochemical Assessment of Damage

PREPARATION OF CELL LYSATES

Ipsilateral samples of sham and ischaemic rat cortex and striatum were isolated and homogenised using a glass Dounce homogeniser (Jencons) by performing 6 'up/down' strokes using a loose-fitting

and 10 'up/down' strokes using a tight-fitting homogeniser on ice. Cells were homogenised in an extraction buffer optimized for subcellular fractionation of rat brain tissue (10mM Tris-Cl (pH 7.4), 0.32M sucrose, 1mM EGTA, 125mM NaCl, 0.1mM DTT, lactacystin (20 μ M), phosphatase inhibitor cocktail (25mM sodium pyrophosphate, 10mM β -glycerolphosphate, 10mM sodium orthovanadate), 20 μ M lactacystin, 1mM PMSF and 10 μ g/mL protease inhibitor cocktail– aprotinin, pepstatin and leupeptin). Whole cell homogenates were passed through 0.35mm gauze to remove unbroken cells, cell debris, blood vessels etc

ISOLATION OF CYTOSOLIC AND MITOCHONDRIAL FRACTIONS

Lysates were maintained at 4°C throughout the subcellular fractionation experiment. Cell debris and nuclei were removed by centrifugation at 1000g for 3 min using a benchtop centrifuge (). The resultant supernatant represented the whole or crude cell lysate. The supernatant was subsequently spun at 10,000g for 20 min to separate the cell lysate into crude cytosolic (supernatant) and mitochondria (pellet) fractions. The crude mitochondria-containing pellet was washed twice (10,000g for 20 min) with SCF buffer and the resultant enriched-mitochondrial fraction re-suspended in detergent containing SCF buffer (2% CHAPS SCF buffer, 30 min) to facilitate mitochondrial membrane solubilisation. The crude cytosolic fraction from the initial spin was subjected to a further centrifugation at 100,000g for 1 h in the ultracentrifuge (TL-100, Beckman-Coulter) to pellet out endoplasmic reticulum and lysosomes. The purified cytosolic (S100) and enriched-mitochondrial fractions were subsequently analysed by SDS-PAGE and Western blotting.

Total lysate, cytosolic and mitochondrial fractions were analysed using Western blotting for changes in pro-apoptotic Bax, Bad and Nix protein expression. Proteins in each fraction (30 μ g) were separated by gel electrophoresis and incubated overnight at 4°C with one of the following rabbit polyclonal primary antibodies: anti-cleaved caspase-3 (1:1000; New England BioLabs), anti-Bax NT (1:500; Upstate), anti-Bad (1:1000), anti-phosphoserine (P)¹³⁶ Bad (1:1000), anti-Akt (1:1000), anti-phosphoserine (P)⁴⁶³ Akt (1:1000; all Cell Signalling Technology) or anti-BNip3L (1:1000; ψ ProSci Inc.). Membranes were incubated with appropriate mouse primary antibodies: anti- β -tubulin (1:1000), or anti- α -tubulin (1:2000) or rabbit polyclonal anti-VDAC (1:2000; all Sigma) to ensure equal loading, protein transfer and fraction purity in crude cell lysate, cytosolic and mitochondrial fractions, respectively. The immunoreactive proteins were visualized using the appropriate horseradish peroxidase-coupled anti-rabbit or anti-mouse immunoglobulin secondary antibody (1:2500, Amersham Life Sciences) and enhanced chemiluminescence (ECL, Amersham Life Sciences).

RESULTS

VOLUME OF DAMAGE

In this series of studies, focal cerebral ischaemia was induced in rats by intraluminal MCA occlusion. This method was shown in Chapter 2, and in other studies (Nagasawa *et al.* 1989, Zea Longa *et al.* 1989), to reliably and reproducibly induce infarcts which are restricted to the MCA territory, encompassing striatal and cortical tissue. Lesion margins were identified by thionin staining, where tissue damage was assessed by loss of thionin staining in the ipsilateral hemisphere 6 h and 24 h after onset of ischaemia. Occlusion of the MCA for 6 h resulted in an area of pallor that was restricted to the striatum and appeared primarily necrotic. No damage was observed in the overlying cortex. In contrast, 24 h of ischaemia produced an extensive infarct restricted to the striatum and cortex (*Figure 6.1*).

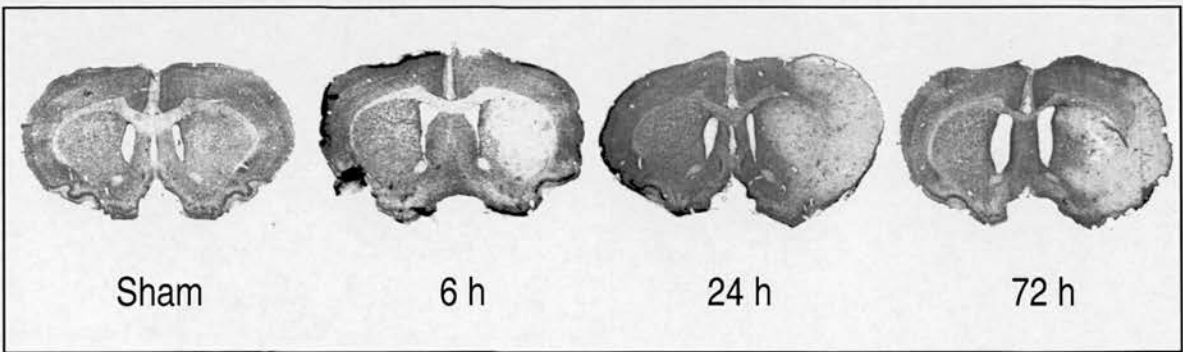


Figure 6.1: Thionin stained brain sections after permanent MCA occlusion in rats.

Histological assessment of damage was performed on thionin stained sections for each time point investigated (n=3). The lesion was restricted to the striatum at 6 h, with expansion into the cortex after 24 h permanent ischaemia. The volume and pattern of cell damaged after 24 h permanent ischaemia was comparable to that observed after 72 h transient (2 h MCA occlusion) ischaemia where gross cell lysis was observed. One representative brain section per treatment group.

The volume and distribution of the lesion after 24 h permanent ischaemia was indistinguishable from that observed at 3 days suggesting the lesion was anatomically mature after 24 h (*Figure 6.2*). Closer examination identified scattered neurones on the boundary and within the infarct after 24 h ischaemia. No gliosis was detected, however many of the remaining cells within the striatum appeared as ‘ghost cells’, resembling the end stages of infarction.

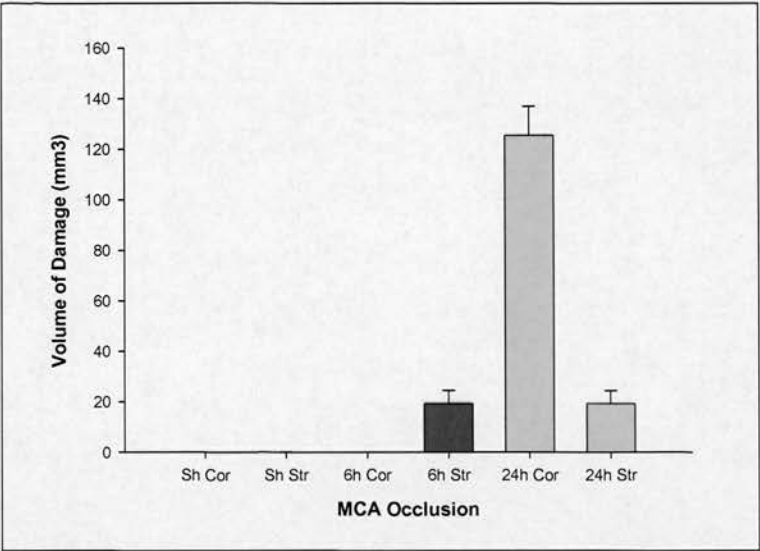


Figure 6.2: Volume of tissue damage observed with increased ischaemic duration.

Coronal brain sections (20µm) were obtained following 6 h and 24 h permanent focal cerebral ischaemia (n=3). Volume of damage was measured as the area of pallor within the lesion boundary, as determined by thionin staining. The volume of striatal damage following 6 h and 24 h permanent ischaemia was $19.21 \pm 5.25 \text{ mm}^3$ and $20.56 \pm 4.67 \text{ mm}^3$, respectively. In contrast, a cortical lesion of $125.46 \pm 5.25 \text{ mm}^3$ was observed after 24 h ischaemia.

In contrast to thionin staining, brain sections of 6 h and 24 h occluded rats re-stained with H&E (nuclear stain) identified numerous cells morphologically resembling neurones in the core (striatum) and penumbra (cortex). After 6 h ischaemia, individual cells of the deeper cortical appeared to be compromised, displaying features of cell shrinkage and pyknosis. However, with increased ischaemic duration (24 h) the density of apoptotic cells increased and encompassed the entire vascular territory - the greatest magnitude of apoptotic response was observed within the core lesion at this time point. Only few remaining neurones were detected with thionin which was in stark contrast to the abundance of apoptotic cells primarily observed in the most severely damaged tissue observed following H&E staining (*Figure 6.3*).

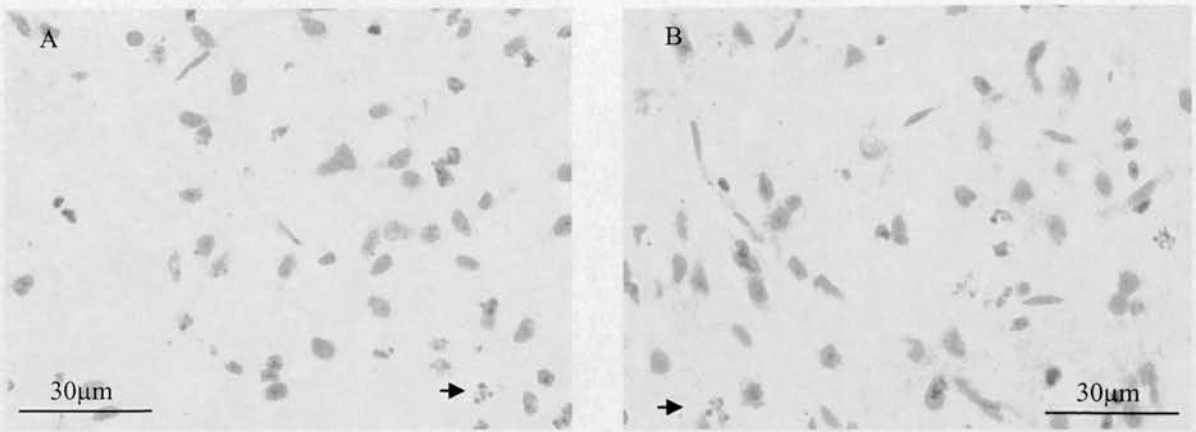


Figure 6.3: H&E staining of the ischaemic lesion after 24 h permanent ischaemia.

Thionin stained sections were subsequently re-stained with haematoxylin and eosin (H&E) to further characterise specific nuclear changes observed with increased ischaemic duration. Data represents sections from an individual brain. Numerous apoptotic-like shrunken cells with apoptotic bodies (arrow) were expressed primarily in the core striatal lesion (A) and also the less severely damaged cortex (B) after 24 h permanent ischaemia.

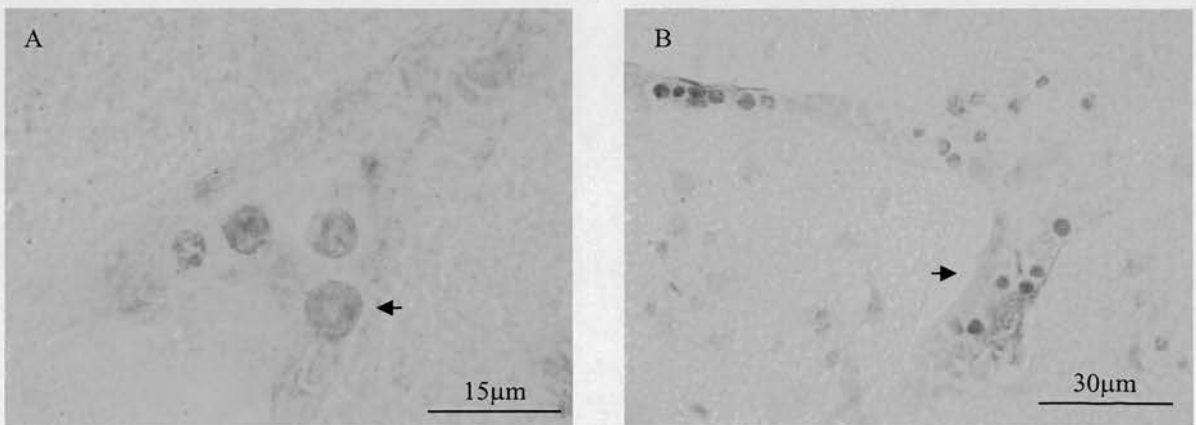


Figure 6.4: Perivascular response in the cortex and striatum after 6 h and 24 h permanent ischaemia.

Using H&E staining, an increased peri-vascular response was observed in the core striatal lesion after 6 h and 24 h permanent ischaemia (A) and in the deeper cortex after 24 h ischaemia (B). Small, round and darkly stained agranular cells (arrow) were observed at this time point, which resembled monocytes.

A perivascular response was observed in the striatum and deeper cortex, which appeared to increase with increased ischaemic duration. Agranular cells, possibly monocytes, with a rounded-like structure and clear cytoplasm were observed within and escaping from blood vessels (Figure 6.4). Infiltration of multi-lobed cells with a horseshoe-like structure, possibly neutrophils, was also observed in the deeper cortex after 24 h (Figure 6.5).

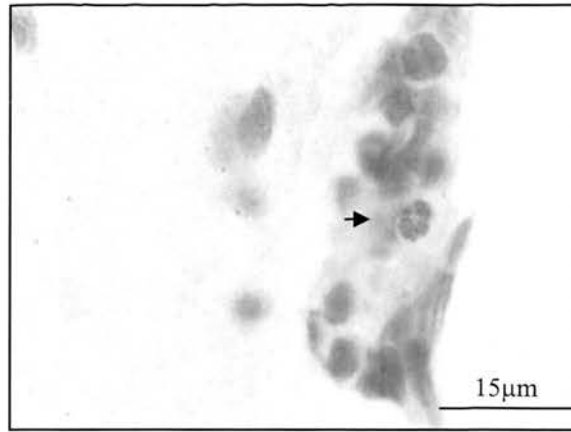


Figure 6.5: Perivascular response in the cortex after 24 h permanent ischaemia.

Infiltration of a second cell-type was observed within the deeper cortex after 24 h permanent ischaemia. Multi-lobed cells, reminiscent of neutrophils were observed at this time point, in the cortex, but not in the less well perfused striatum. (Identical H&E stained section as examined in *Figure 6.4*).

BCL-2 FAMILY RESPONSE TO PERMANENT ISCHAEMIA

Initial biochemical studies in transiently occluded stroke tissue (*see Chapter 2*), identified that all three proteins, as well as the 30 kDa Nix-like immunoreactive protein, were endogenously expressed in the rat striatum and cortex. In this chapter, lysates from sham and permanent (6 h and 24 h) MCA occluded rats were analysed for changes in pro-apoptotic Bax, Bad and Nix protein expression. Increased Bax expression was observed in the striatum, but not cortex, after 24 h ischaemia (*Figure 6.6A*). A progressive increase in Nix expression was detected in the striatum between 6 and 24 h ischaemia (*Figure 6.6B*), while a delayed upregulation/de novo synthesis of the 30 kDa Nix-like protein was observed in the core striatal lesion only after 24 h. Changes in Bad protein expression could not be detected in the present study. Since *in vitro* studies (*see Chapters 3 and -4*) reported upregulation of Bax, Nix and the 30 kDa protein following apoptotic stimuli, these findings support a role for the Bcl-2 family proteins Bax, Nix and the 30 kDa Nix-like proteins in the progression of ischaemic cell damage.

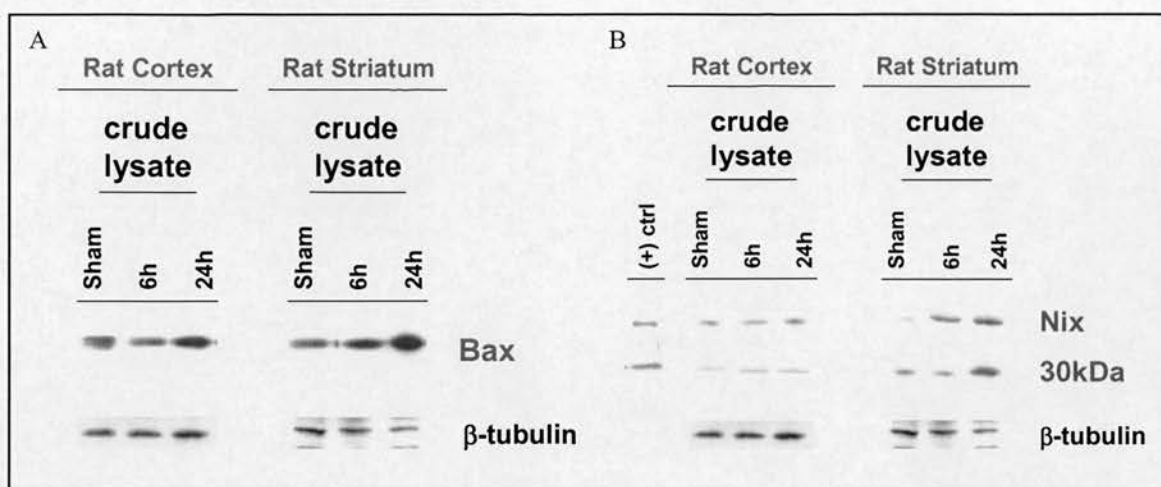


Figure 6.6: Changes in Bax and Nix protein expression after 6 h and 24 h permanent ischaemia.

Cell lysates from sham, 6 h and 24 h permanent MCA occluded rats were analysed for changes in apoptotic Bax (A) and Nix (B) protein expression with increased ischaemic duration. Bax upregulation was observed in the striatum after 24 h permanent ischaemia. A progressive increase in Nix protein expression was observed between 6 and 24 h ischaemia in the striatum, and after 24 h in the cortex. A second immunoreactive band, which migrated as an apparent 30 kDa protein, was observed in the striatum after 24 h permanent ischaemia. Nix antibody specificity was confirmed using K562 cell lysates as a positive control. β-tubulin confirmed equal loading (7 pooled animals per treatment; n=7).

In vitro studies (see Chapters 3, -4 and -5) suggested that subcellular distribution of pro-apoptotic Bcl-2 family proteins was a more suitable measure of protein activation. Subcellular fractionation was optimized in rat brain tissue. Cytosolic and mitochondrial fractions from sham and ischaemic (6 h or 24 h) rat brains were analysed for changes in the subcellular distribution of Bax (Figure 6.7A), Nix and the 30 kDa Nix-like protein (Figure 6.7B) with increased ischaemic duration. Bax appeared primarily cytosolic, and to a lesser extent, mitochondrial localised in the striatum and cortex of sham animals. In contrast, Nix was detected in each cell fraction of sham animals and the 30 kDa Nix-like protein was only detected in the mitochondria. Bax upregulation was observed in the striatum, and less so in the cortex after 24 h ischaemia. However, concomitant Bax translocation towards the mitochondria was only observed in the core striatal lesion (Figure 6.7A). Progressive upregulation of mitochondrial Nix was observed in each brain region after 6 h and 24 h ischaemia. Upregulation of cytosolic Nix was also detected in the cortex and striatum after 24 h (Figure 6.7B). Although cytosolic levels of the 30 kDa Nix-like protein were reduced in the cortex and striatum at 6 h, mitochondrial accumulation was not observed. Overall, increased levels of cytosolic and mitochondrial Bax were observed in the striatum at 24 h, indicative of Bax translocation to mitochondrial, with *de novo* protein synthesis. The subcellular distribution for Bax did not change in the cortex at any time point. In contrast, mitochondrial accumulation of Nix and the 30 kDa Nix-like protein was observed with increased ischaemic duration, in both the cortex and striatum. These findings support a model for an increased pro-apoptotic response with increased severity of ischaemic insult.

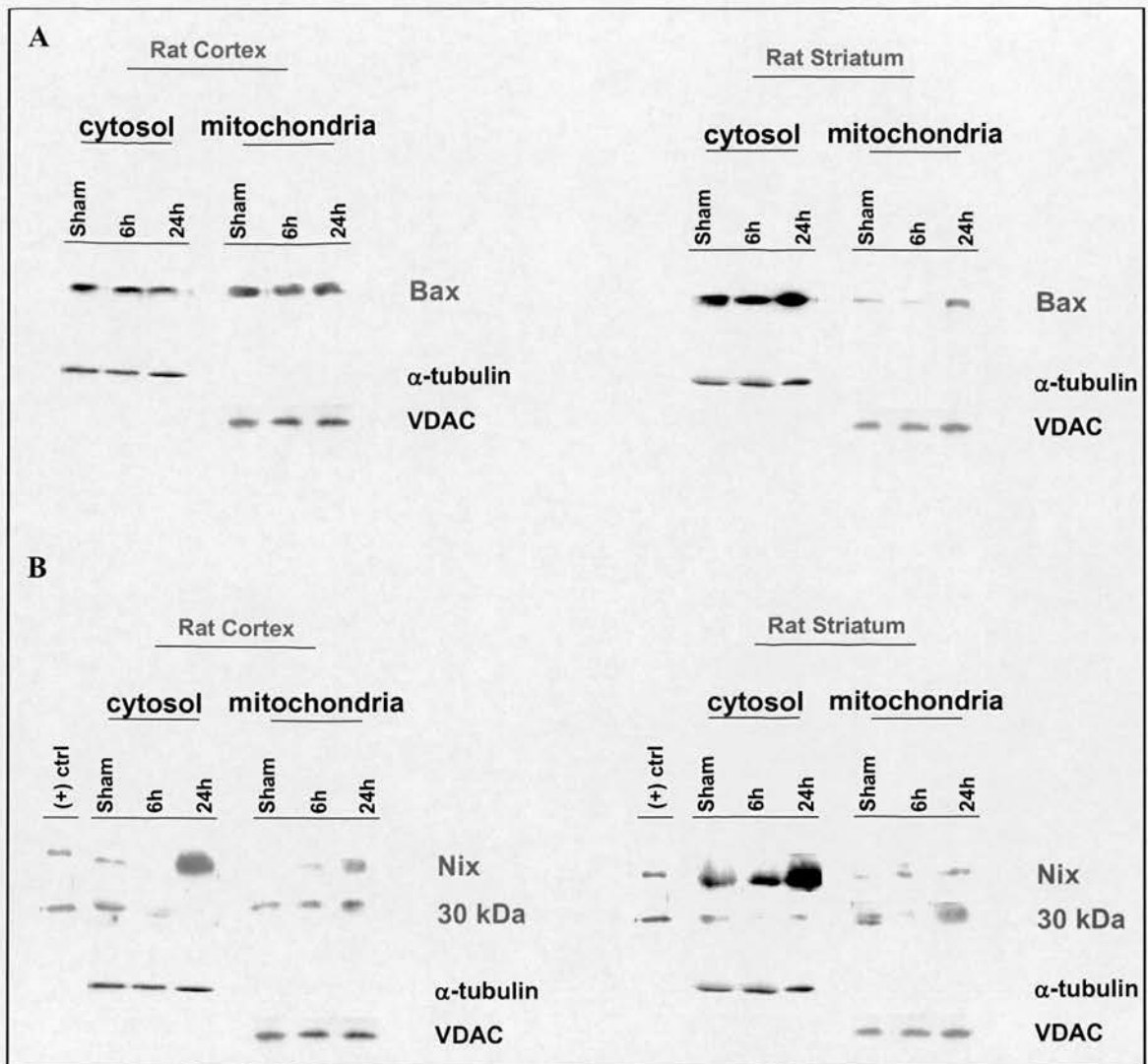


Figure 6.7: Changes in the subcellular distribution of Bax and Nix with increased ischaemic duration.

Cytosolic and mitochondrial fractions were obtained from sham, 6 h and 24 h occluded rat brains and assessed for changes in the subcellular distribution of Bax (A) and Nix (B) (n=7). Bax and Nix appeared as primarily cytosolic proteins, but were also detected in the mitochondria of sham animals (mitochondrial membrane of Bax cortex overdeveloped to enable detection). The 30 kDa protein was detected in approximately equal amounts in both fractions (B). Cytosolic upregulation and mitochondrial accumulation of Bax was observed in the striatum after 24 h. Cortical distribution of Bax did not change. Increased mitochondrial accumulation of Nix was observed in the cortex and striatum between 6 h and 24 h. A marked upregulation of cytosolic Nix was detected after 24 h in both brain regions. In contrast, reduced cytosolic levels of the 30 kDa protein were observed within 6 h ischaemia, however, mitochondrial accumulation of this protein was only detected in the striatum after 24 h. VDAC and β -tubulin confirmed equal loading and fraction purity.

Kitagawa *et al.* (1999) first reported an Akt-mediated survival response following permanent focal cerebral ischaemia, where increased Akt immunoreactivity was reported in the cortex after 3 h ischaemia, but decreased by 24 h. Bad activity was not reported in that study. *In vitro* studies (see Chapter 4) identified P⁴⁷³ Akt in both the cytosol and mitochondria of healthy SH-SY5Y neuroblastoma cells, with loss of staining in both fractions following an apoptotic stimulus. This

study was therefore designed to investigate the phosphorylation status of both Akt and Bad as well as changes in subcellular distribution of each protein as a measure of protein activity following permanent ischaemia. Cell lysates from sham and MCA occluded (6 h and 24 h) rats were initially assessed for changes in protein expression and phosphorylation status of Bad and Akt. Bad (Figure 6.8) and Akt immunoreactivity (Figure 6.10) did not appear to change in either brain region after 6 h ischaemia. However, when isolated into enriched cytosolic and mitochondrial fractions changes were observed for each of these proteins. For example, cytosolic levels of both total and P¹³⁶ Bad increased in both brain regions after 6 h ischaemia (Figure 6.9), supporting an early Akt-mediated Bad survival response.

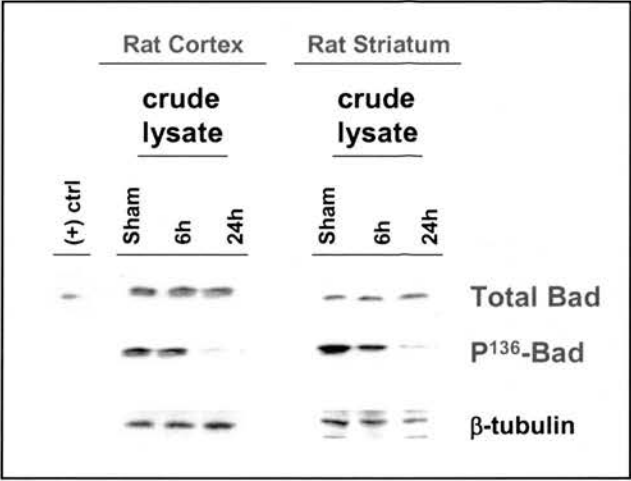


Figure 6.8: Bad protein expression and phosphorylation status with increased ischaemic duration
Total cell lysates from sham, 6 h and 24 h permanent MCA occluded rats were analysed for changes in pro-apoptotic Bad and P¹³⁶ Bad protein expression (n=7). P¹³⁶ Bad immunoreactivity decreased in both the cortex and striatum after 24 h permanent ischaemia. In contrast, total Bad expression did not change at any time point investigated. Specificity was confirmed using peptide supplied with Bad antibody. β-tubulin confirmed equal loading.

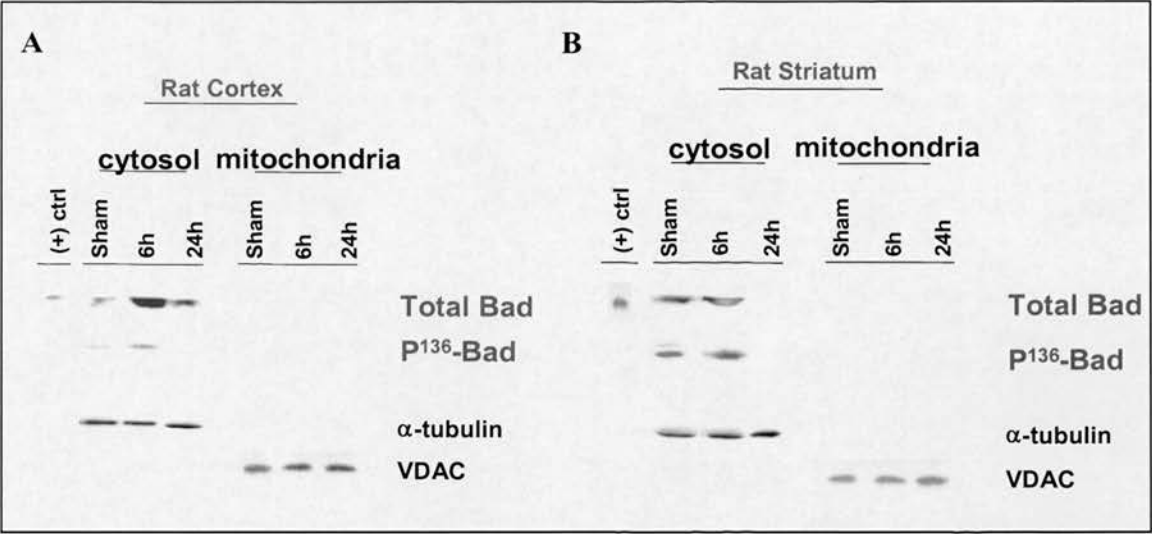


Figure 6.9: Assessment of Bad and P¹³⁶Bad subcellular distribution after 6 h and 24 h permanent ischaemia.

Cytosolic and mitochondrial fractions were obtained from sham, 6 h and 24 h MCA occluded rat brains and assessed for changes in the subcellular distribution of Bad (n=7). Bad appeared cytosolic and phosphorylated in sham animals. Early upregulation of total and P¹³⁶Bad was observed in the cytosolic fraction of both the striatum (A) and cortex (B) after 6 h permanent ischaemia. With increased ischaemic duration (24 h) total and P¹³⁶Bad immunoreactivity was observed to decrease in each brain region. However, a concomitant mitochondrial accumulation of dephosphorylated Bad was not observed. Antibody specificity was confirmed using a Bad peptide supplied with the Bad antibody. VDAC and α-Tubulin confirmed fraction purity and equal loading.

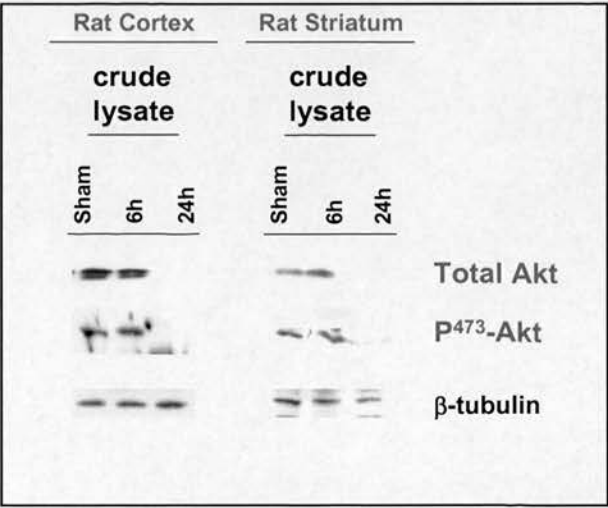


Figure 6.10: Akt and P⁴⁷³ Akt protein expression after 6 h and 24 h permanent ischaemia in rats.

Total cell lysates from sham, 6 h and 24 h permanent MCA occluded rats were analysed for changes in Akt and P⁴⁷³ Akt immunoreactivity (n=7). Loss of all detectable Akt and P⁴⁷³ Akt was observed in both the cortex and striatum after 24 h ischaemia. B-Tubulin confirmed equal loading.

A novel Akt signalling response was also observed in the cortex and striatum at this time point, where P^{473} Akt translocated from the cytosol to mitochondria (Figure 6.11). In contrast, increased ischaemic duration (24 h) was associated with reduced total Bad expression, with loss of detectable phosphorylated Bad (Figure 6.9) and Akt (Figure 6.11) immunoreactivity. Dephosphorylation of these two proteins coincided with increased cleaved caspase-3 immunoreactivity (Figure 6.12) and morphological features of apoptotic cell death (Figure 6.3). Since *in vitro* data (Chapter 4) supports a model of for dephosphorylation and translocation of cytosolic Bad towards the mitochondria following an apoptotic stimulus, it was unclear why mitochondrial accumulation Bad could not be detected in these *in vivo* studies and warrants further investigation.

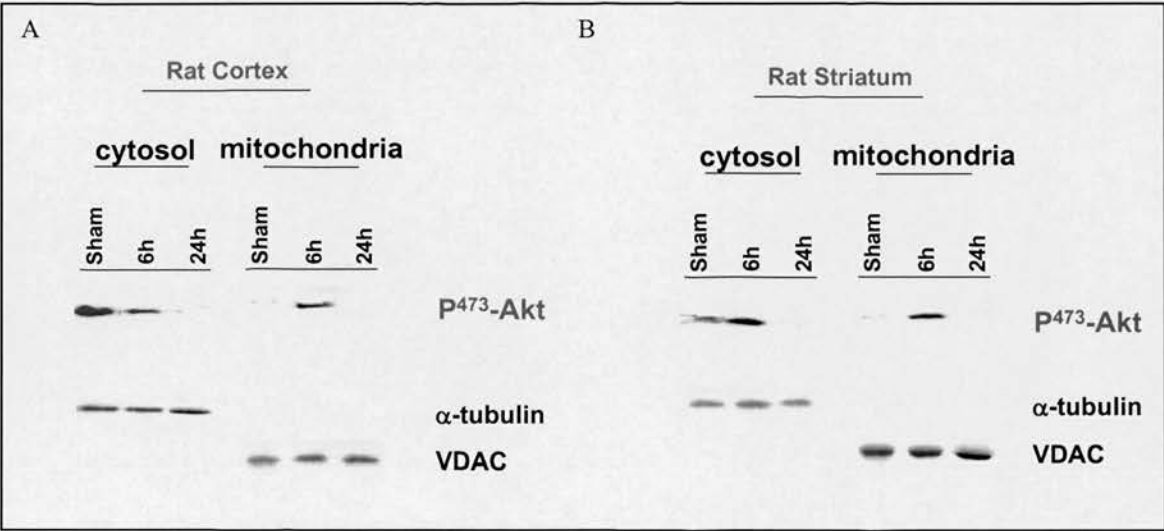


Figure 6.11: Assessment of P^{473} Akt subcellular distribution after 6 h and 24 h permanent ischaemia. Cytosolic and mitochondrial fractions were obtained from sham, 6 h and 24 h MCA occluded rat brains and assessed for changes in the subcellular distribution of P^{473} Akt (n=7). P^{473} Akt was observed as a primarily cytosolic, but also mitochondrial-associated protein in sham animals. Early mitochondrial accumulation of P^{473} Akt was observed in both the cortex and striatum after 6 h ischaemia. However, cytosolic levels of P^{473} Akt were unaltered at this time point. Loss of P^{473} Akt immunoreactivity was observed in both brain regions with increased ischaemic duration (24 h).

An increased pro-apoptotic Bcl-2 family response was observed for each Bcl-2 family protein investigated, with increased ischaemic duration. This was observed primarily in the core striatal lesion. Caspase-3 immunoreactivity was investigated because reports suggest this protein to be a critical effector in the orchestration and dismantling of apoptotic cell death. In support of the aforementioned histological and biochemical findings detailed in this chapter, increased total (Figure 6.12A) and cytosolic (Figure 6.12B) cleaved caspase-3 immunoreactivity was observed in the striatum at 6 h, and both the striatum and cortex following 24 h permanent ischaemia.

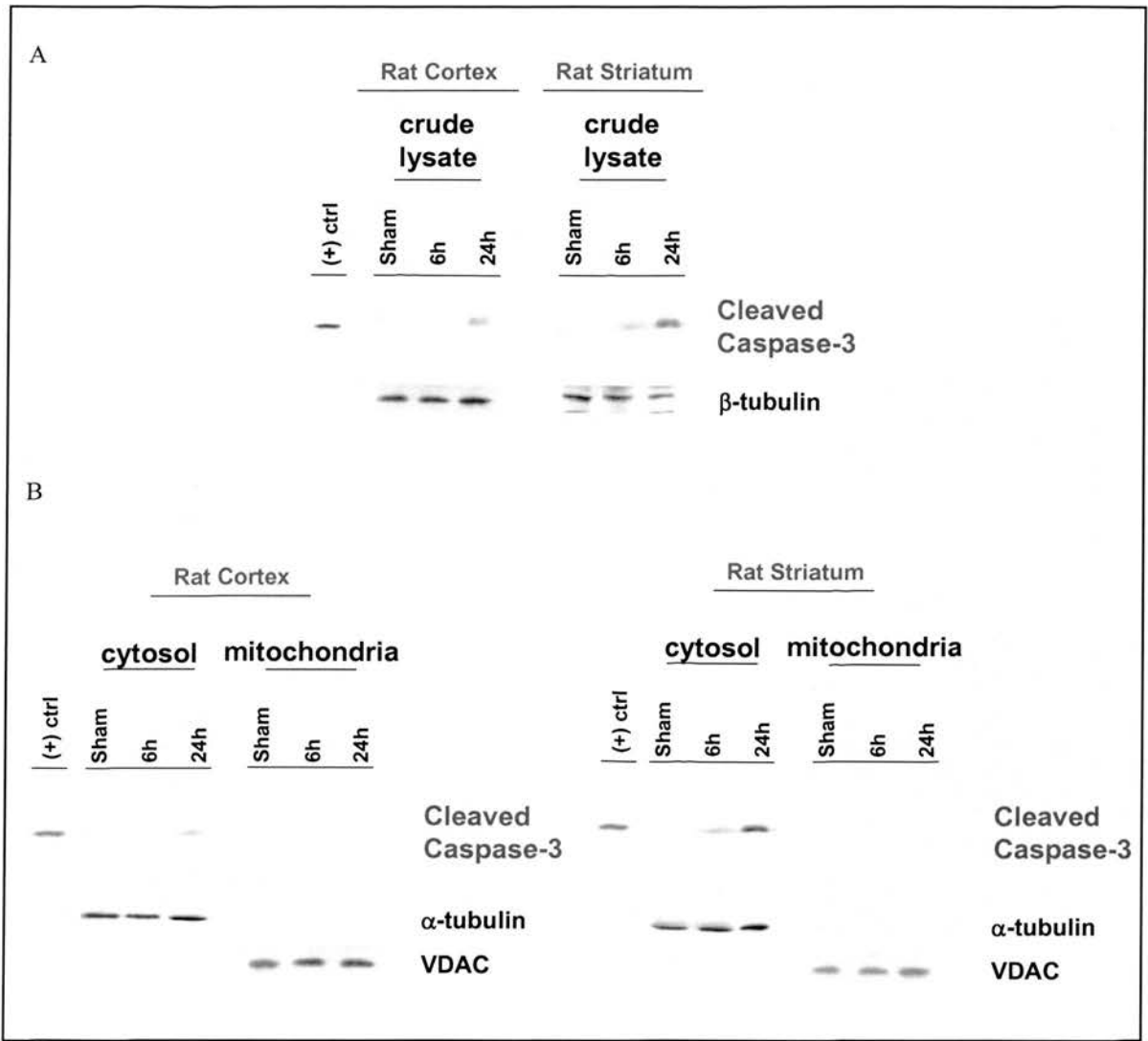


Figure 6.12: Cleaved caspase-3 expression and subcellular distribution after permanent ischaemia.

Total cell lysates were analysed for changes in cleaved caspase-3 protein expression after 6 h and 24 h permanent ischaemia (n=7). A progressive increase in cleaved caspase-3 expression was observed between 6 and 24 h permanent ischaemia in the striatum, and after 24 h in the cortex (A). Cytosolic and mitochondrial fraction was obtained to investigate putative changes in the subcellular distribution of this protein. Cleaved caspase-3 immunoreactivity was restricted to the cytosolic fraction in each brain region (B). Apoptotic rat primary cerebellar granule cells (see Chapter 2) confirmed antibody specificity.

DISCUSSION

Increased Bax mRNA and protein expression was previously reported within the ischaemic hemisphere after 6 h permanent ischaemia in rats (Gillardard *et al.* 1996). However, the role of Bad and Nix protein activity in focal cerebral ischaemia had not been investigated. Also, few studies had correlated biochemical changes with histological damage. It was therefore proposed that

characterisation of the spatiotemporal profile of Bax, Bad and Nix changes with increased ischaemic duration would provide a more detailed understanding of the role, if any, of this protein family in the pathophysiology of focal cerebral ischaemia. Initial studies were designed to investigate histological damage following 6 h and 24 h permanent ischaemia, with the aim of subsequently correlating histological damage with biochemical changes in Bax, Bad and/or Nix protein activity. A lesion restricted to the striatum was observed after 6 h ischaemia. In contrast, lesion expansion was observed after 24 h ischaemia, where a cortical and striatal lesion encompassing the entire MCA territory was reported. At this latter time point, only few surviving neurones could be detected in the core lesion using thionin staining, where many 'ghost-like' cells, considered to mark the final morphological characteristics of necrosis, were observed. In contrast, H&E staining of these brain sections revealed that many more cells within both the core lesion (striatum and deeper cortex) and the penumbra (cortex). In contrast, these cells appeared shrunken, with evidence of pyknotic nuclei, apoptotic body formation, and the morphology of neurones. Interestingly, detection of these apoptotic-like cells appeared to increase with increased ischaemic duration and was most prevalent in the core striatal lesion after 24 h permanent ischaemia; a time point conventionally considered to produce a well-demarcated and primarily necrotic lesion (Siesjö *et al.* 1992a, Lipton *et al.* 1999). These preliminary findings suggested that apoptotic mechanisms might indeed be involved in lesion expansion.

Initial stroke studies (*see Chapter 2*) assessed changes in total protein expression as a measure of pro-apoptotic Bcl-2 family activity. Subsequent characterisation of *in vitro* Bax, Bad and Nix activation (*see Chapters 3, -4 and -5*), confirmed that assessment of subcellular distribution was a more sensitive measure of changes in protein activity. Cell lysates from sham, 6 h and 24 h treatment groups were separated into cytosolic and enriched mitochondrial fractions and analysed for changes in Bax, Bad and Nix protein activity. In this study, an early pro-apoptotic Bcl-2 family response might have been mediated through alterations in the subcellular distribution and activation of Nix and Akt-regulated inactivation of Bad. In contrast, pro-apoptotic Bax, Bad, Nix and the Nix-like protein responses were observed with increased ischaemic duration (24 h), and this appeared to correlate with increased cleaved caspase-3 immunoreactivity and ischaemic damage. Furthermore, using subcellular fractionation as a tool to measure Bcl-2 family activity, a pro-apoptotic response was reported for each protein investigated. *In vitro* studies have reported Bax to be a major pro-apoptotic mediator in apoptotic cell death (Antonsson *et al.* 2001, Kroemer *et al.* 2001). Translocation of Bax to the mitochondria has been reported to constitute a critical event in the induction of neuronal apoptosis induced by several stimuli (Xiang *et al.* 1998, Cregan *et al.* 1999, Martinou *et al.* 1999, Li *et al.* 2002). Bax appeared as a primarily cytosolic, and to a lesser extent, mitochondrial protein in sham animals. These findings are consistent with *in vitro* data (*see Chapter 3*). However, neither Bax protein expression nor subcellular distribution appeared to change in either brain region after 6 h ischaemia. These findings are in contrast to a previous study which reported Bax upregulation, primarily in the cortex 7 h after onset of 1h transient ischaemia (Gillardon *et al.* 1996). However,

Gillardon's study observed a lesion encompassing the cortex and striatum. In contrast, the ischaemic lesion reported in this chapter was restricted to the striatum, and therefore such a Bax response might not be expected in the cortex in present study. However, increased ischaemic duration (24 h) was associated with upregulation of cytosolic Bax, primarily in the core striatal lesion, with slight induction in the less damaged cortex. This might be indicative of an increased pro-apoptotic response, which correlated with the greatest magnitude of ischaemic damage and morphological features of apoptotic cell death in the striatum after 24 h permanent ischaemia.

The role of Nix in stroke pathology was also investigated. Although initial *in vitro* studies (see Chapter 5) demonstrated that BNip3 protein activity could not be investigated due lack of antibody specificity, these experiments identified a 30 kDa immunoreactive band which might represent the monomer of BNip3, or alternatively, a novel Nix-like protein. Therefore, this chapter also addressed whether or not the 30 kDa Nix-like protein could be detected, and if so, was this protein responsive to ischaemia in rat brain tissue. Initial studies reported a progressive upregulation of Nix in the core striatal lesion, and less so in the cortex, with increased ischaemic duration. As predicted, a comparatively delayed response was observed for the Nix-like protein, where upregulation of this protein was reported in both brain regions after 24 h. Cytochrome c release has been reported an early event, occurring within the first few hours during permanent cerebral ischaemia (Li *et al.* 2002). However, based on data presented in this chapter, it seems unlikely that such an event would be mediated by Bax, since Bax translocation to the mitochondria was not detected within this time point. It was therefore proposed that the observed Nix response might mediate such an event in the present study. Interestingly, although *in vitro* studies identified the Nix-like protein as a mitochondrial-associated protein in untreated human SH-SY5Y neuroblastoma cells (see Chapter 5); this protein was also detected in the cytosolic fraction of sham rat cortex and striatum. Nix appeared primarily cytosolic, similar to *in vitro* studies, but was also detected in the mitochondrial fraction of sham animals. This might simply reflect higher endogenous protein expression levels in rat brain compared to the cell lines used. As predicted, Nix upregulation in the striatum after 6 h ischaemia was accompanied by translocation of this protein to the mitochondria. Perhaps most intriguing was the finding that Nix translocation to the mitochondria was also observed in the cortex at a time where histological damage could not be detected. Mitochondrial accumulation of Nix further increased in both brain regions with increased ischaemic duration. Also, a marked upregulation / *de novo* synthesis of Nix was observed in the cytosolic fraction of each brain region. These findings however, might be met with caution where some studies (Sims & Anderson 1999), but not all (Back *et al.* 2000, Hata *et al.* 2000b) would argue that energy levels within the ischaemic brain at these time points might be insufficient to permit Nix protein synthesis.

BNip3 has been reported to localise primarily to mitochondria and other cytoplasmic membranes (Boyd *et al.* 1994) and is widely expressed in a number of mouse and human tissues (Chen *et al.* 1996). In contrast, few reports have investigated Nix protein expression, although studies have also reported Nix to be a primarily mitochondrial-associated protein (Chen *et al.* 1999). In contrast to Nix-mediated cell death which is associated with cytochrome c release, caspase-3 cleavage (Ray *et al.* 2000), both apoptotic and necrotic cell death mechanisms have been attributed to BNip3 (Yasuda *et al.* 1998, Ray *et al.* 2000, Vande Velde *et al.* 2000). A recent study reported that BNip3 induces a necrotic-like cell death after mitochondrial integration into the OMM. This event was associated with rapid PTP opening, generation of reactive oxygen species and cytochrome c release with delayed detection of nuclear changes (Vande Velde *et al.* 2000). Based on these studies, and *in vitro* data (Chapter 5) it was hypothesised that if the Nix-like protein did indeed represent a BNip3 monomer, it might be anticipated that Nix and the 30 kDa Nix-like protein would have different spatial and/or temporal profiles to permanent focal cerebral ischaemia. For example, it was proposed that Nix might mediate the early pro-apoptotic response involving activation of an energy-consuming mechanism. It was also proposed that activation of the Nix-like protein, a less energy-consuming process, might mediate a delayed response with increased ischaemic duration. Indeed, compared to the early upregulation and mitochondrial accumulation of Nix observed in the striatum, increased ischaemic duration was accompanied by a delayed mitochondrial accumulation of the Nix-like protein only after 24 h ischaemia. It should be noted that, while an early reduction in cytosolic levels of this protein was observed in each brain region, a concomitant mitochondrial accumulation was not observed after 6 h ischaemia. Since, early studies have reported the ability of BNip3 and Nix to localise to both the nucleus and endoplasmic reticulum (Boyd *et al.* 1994) and that both organelles have been implicated in apoptotic cell death (Nutt *et al.* 2002), it might be proposed that the Nix-like protein, did indeed mediate an early response to ischaemia, but that this protein's site of action might not necessarily have been the mitochondria. Investigation of such a proposed mechanism of action was outside the limits of this thesis, but warrants further investigation. However, activation of both Nix and the 30 kDa Nix-like protein after 24 h ischaemia might account for the mixed apoptotic and necrotic morphology reported in this study, and in others (Lipton *et al.* 1999, Nicotera *et al.* 1999b).

Several studies have suggested a protective role for growth factors or neurotrophic factors in ischaemic brain injury (Tanaka *et al.* 1995, Schabitz *et al.* 1997, Kitagawa *et al.* 1998, Kitagawa *et al.* 1999). Growth factor activation of the PI3-K/Akt signalling pathway has been reported to phosphorylate Bad on serine residue-136 and to suppress apoptosis (Philpott *et al.* 1997). Changes in P⁴⁷³ Akt have been observed an early event during focal ischaemia, however attempts to correlate these changes with changes in Bad activity proved unsuccessful (Friguls *et al.* 2001, Noshita *et al.* 2001). Therefore this chapter investigated whether (i) a similar Akt-mediated survival mechanism occurred in this rat model of permanent focal cerebral ischaemia, and if so (ii) was this event mediated by inhibition of Bad. No significant changes in Bad or P¹³⁶ Bad protein expression were detected in

whole cell lysates after 6 h ischaemia. In keeping with previous *in vitro* reports (and data from Chapter 5), total and P¹³⁶ Bad were only detected in the cytosol of sham animals. In contrast, an early increase in cytosolic levels of total Bad and P¹³⁶ Bad was detected in the cortex and striatum after 6 h ischaemia. These findings suggest that subtle changes in Bad activity might only be detectable in enriched cell fractions.

In vitro data (see Chapter 4), supported a model whereby Akt phosphorylates Bad on serine residue-136. In turn, P¹³⁶ Bad is localised to the cytosol of healthy cells, bound in an inactive state to scaffolding proteins, such as 14-3-3 proteins (Zha *et al.* 1996, Muslin *et al.* 2000). Following an apoptotic stimulus, dephosphorylated Bad translocates to the mitochondria and promotes apoptotic cell death, possibly through dimerisation with anti-apoptotic Bcl-X_L and thus displacement and indirect activation of pro-apoptotic Bax (Zha *et al.* 1996). An Akt-mediated survival response has been reported following global (Yano *et al.* 2001) and focal cerebral ischaemia (Kitagawa *et al.* 1999, Friguls *et al.* 2001, Noshita *et al.* 2001a), as well as being implicated in preconditioning after global ischaemia (Yano *et al.* 2001). However, these studies did not investigate changes in the subcellular distribution of the protein as a measure of activity. Subsequent studies therefore investigated the role of Akt in regulation of the observed Bad response. Similarly, total Akt and P⁴⁷³ Akt protein expression levels did not change in response to 6 h ischaemia. However, consistent with *in vitro* studies (see Chapter 4), P⁴⁷³ Akt was observed in both the cytosolic and mitochondrial fractions of sham animals. Translocation of P⁴⁷³ Akt to the mitochondria / upregulation of both mitochondrial and cytosolic P⁴⁷³ Akt were observed at this time. This is the first study to demonstrate re-distribution of both Akt and Bad following focal cerebral ischaemia. Moreover, this is the first study to report such an intrinsic survival mechanism within the core and penumbra regions of the ischaemic lesion. Other studies have failed to correlate an observed Akt-mediated survival mechanism with concomitant Bad changes (Kitagawa *et al.* 1999, Ouyang *et al.* 1999, Friguls *et al.* 2001, Janelidze *et al.* 2001, Noshita *et al.* 2001). However, these studies assessed total protein expression rather than subcellular distribution underscoring the importance of choosing the most appropriate method of assessing protein activity. Initial studies also investigated the effects of increased ischaemic duration on P¹¹² Bad protein expression and subcellular distribution (not reported). Although the lack of a specific P¹¹² Bad response was initially attributed to antibody problems, other studies have also failed to report changes in this protein under similar ischaemic conditions (Shibata 2002). Therefore, under these conditions, the phosphorylation status of serine residue-136 might better reflect Bad activation status. Altogether, these findings support the use of subcellular fractionation as a measure of protein activity and emphasize the need to characterise Bcl-2 family protein-specific changes with increased ischaemic duration.

Increased ischaemic duration was associated with loss of total and P⁴⁷³ Akt immunoreactivity in the cytosol and mitochondria of both cortex and striatum. A concomitant reduction in cytosolic levels of total and P¹³⁶ Bad was observed after 24 h ischaemia, indicative of a pro-apoptotic response (Wang *et al.* 1999). However, in contrast to *in vitro* studies (Chapter 4), concomitant mitochondrial accumulation of dephosphorylated Bad was not detected. Supporting these findings, other studies reported a reduction of Akt or P⁴⁷³ Akt immunoreactivity after 24 h ischaemia (Kitagawa *et al.* 1999, Friguls *et al.* 2001, Noshita *et al.* 2001). Interestingly, Bax translocation towards the mitochondria was not observed until 24 h ischaemia – which correlated with loss of mitochondrial-associated P⁴⁷³ Akt. These findings support available *in vitro* data which suggests that Akt might indirectly regulate the subcellular distribution of Bax (Yamaguchi *et al.* 2001, Tsuruta *et al.* 2002). For example, constitutive activation and over-expression of Akt was reported to inhibit Bax conformational changes and translocation in DNA damaged FL5.12 cells (Yamaguchi *et al.* 2001) and staurosporine-treated HeLa cells (Tsuruta *et al.* 2002). Furthermore, PI3'K inhibitors, LY294002 and wortmannin, were observed to reverse the effects of Akt over-expression on Bax subcellular distribution. Since Bax does not appear to contain Akt phosphorylation sites, it was considered unlikely that Akt directly might regulate Bax translocation (Yamaguchi *et al.* 2001).

The exact mechanism by which phosphorylated Akt mediates events following ischaemia remains unclear. However, recent evidence suggests that Akt might also protect cells from death receptor activation through NF- κ B activation (Hatano *et al.* 2001) or through inhibition of caspase-8 (Chen *et al.* 2001). Interestingly, caspase-8 and caspase-3 expression was reported in different sub-populations of cortical neurones in one model of permanent ischaemia (Namura *et al.* 1998). Therefore, the Akt response described in this chapter might be expressed in a sub-population of resistant neurones. This would explain why pro-apoptotic (increased caspase-3, Bax and Nix expression) and pro-survival (Akt redistribution and Bad phosphorylation) responses were observed within the same brain region. Unfortunately, since the entire MCA territory was used for biochemical analysis of striatal and cortical response, such an accurate characterisation of each response was not possible. Whether the intrinsic Akt-mediated survival response reported in this chapter was mediated through indirect inhibition of Bax, Bad, death receptor activation or a combination of these events requires warrants further investigation. Furthermore, whether the observed survival mechanism prevents cell death in a sub-population of particularly resistant cells, or whether this mechanism, like some neuroprotectants (J. McCarter 2001) merely delays the progression of cell death is unclear. Recent studies confirm that several pro-apoptotic Bcl-2 family members amplify cell death signals or directly affect apoptosis through the mitochondrial pathway by promoting cytochrome c release and subsequently activating terminal caspases, especially caspase-3 (for reviews see Budihardjo *et al.* 1999, Kroemer and Reed 2000). Since each of the Bcl-2 family proteins investigated displayed an increased pro-apoptotic response with increased ischaemic duration, subsequent studies investigated the role of this protein family in apoptotic cell death and lesion expansion. Increased levels of cleaved caspase-3

immunoreactivity were used to confirm apoptosis. Caspase-3 activation has been reported with varying time course and amplitude, according to the severity of the ischaemic insult (Hara *et al.* 1996, Endres *et al.* 1998, Himi *et al.* 1998). Caspase-3 expressing neurones have been reported to display a condensed cytoplasm and nucleus (Velier *et al.* 1999, Benchoua *et al.* 2001). Further evidence supporting a specific role for caspase-3 in lesion expansion, was the finding that administration of caspase-3 inhibitors reduced infarct volume within the core lesion; an area conventionally considered not to be amenable to neuroprotection (Velier *et al.* 1999, Guegan & Sola 2000, Benchoua *et al.* 2001). This suggests that the effector caspase-3 might be involved in dismantling of the cell following either an apoptotic and/or necrotic stimulus. Interestingly, loss of Akt immunoreactivity after 24 h ischaemia in this study was associated with concomitant increases in cleaved caspase-3 immunoreactivity, where the greatest magnitude of caspase-3 response was observed in the core striatal lesion after 24 h permanent ischaemia. Histological and biochemical data therefore supports a role for the pro-apoptotic Bcl-2 family proteins Bax, Bad, Nix and the Nix-like protein in the pathophysiology of stroke.

Based on data reported in this chapter, it might be proposed that *BH3 domain-only* proteins respond to permanent ischaemia prior to activation of the *multidomain* family members. For example, redistribution of Bad, Nix and the Nix-like protein was observed after 6 h ischaemia in both the cortex and striatum. In contrast, Bax distribution was comparable to sham animals at this time point. Minimal caspase-3 immunoreactivity observed in the core striatal lesion might therefore be attributed to the increased levels of mitochondrial-associated Nix, rather than Bax. However, whether Nix can directly promote cytochrome c release, or whether this protein requires a *multidomain* member, such as Bax, has not been determined. Notably, an increased pro-apoptotic Nix response was reported in the cortex after 6 h ischaemia; a time point where no histological damage was observed. Therefore, the Bcl-2 family, specifically Nix might be implicated in lesion expansion, at least in the model investigated. Together, these findings support a model where an early response might be mediated by BH3-only proteins, in contrast to a combined BH3-only and multidomain response with increased ischaemic duration. According to this hypothesis, the BH3-only proteins might play a decisive role in the transduction and integration of pro-apoptotic signals following focal cerebral ischaemia. Unfortunately, difficulties in assessing protein changes in the ischaemic brain have resulted in only a few published reports being available. Consequently, lack of comparable studies in literature made confirmation of data presented in this chapter difficult. Interestingly, such responses were only readily identifiable by assessing changes in the subcellular distribution of Bax, Bad, Nix and the Nix-like protein, supporting the use of subcellular fractionation to assess Bcl-2 family protein activity.

One caveat to this proposed mechanism regards the unresolved issue; which cell type (s) expressed these Bcl-2 family protein responses? Thionin staining of brain sections after 24 h ischaemia only

detected a few remaining neurones within the core ischaemic lesion, with slightly better cortical preservation. In contrast, H&E staining of the same sections revealed many more surviving cells, with a neuronal-like morphology, in both the core striatal lesion and less severely damaged cortex. However, whether sufficient neurones were available to support such an observed pro-apoptotic response, which appeared with greatest magnitude in the most severely damaged tissue, could not be determined with tools available at the time of study. Interestingly, a perivascular pro-inflammatory response was observed in both the striatum (6 h and 24 h) and cortex (24 h) which appeared to progress with increased ischaemic duration and cell damage. Relatively small, rounded and darkly stained agranular cells, reminiscent of monocytes (white blood cells) accumulated within, and escape from blood vessels. A progressive increase in detection of large foamy cells, reminiscent of macrophages (differentiated monocytes), with a speckled appearance was also observed in the core ischaemic lesion. These findings are in keeping with available data that suggests macrophages facilitate removal of cell debris (Turner *et al.* 2003). However, in contrast to data presented in this study, most studies have reported a delayed pro-inflammatory response to focal cerebral ischaemia, in which peak levels of inflammatory cells have been reported between 24 h and 72 h ischaemia (del Zoppo *et al.* 2000, Endres *et al.* 2002, Danton *et al.* 2003). However, since numerous variables might exist between research groups as well as between individual researchers, comparison of histological and biochemical data from a given treatment group of animals might provide a more accurate evaluation of the pathophysiological mediators in a given model of focal cerebral ischaemia. Interestingly, an increased inflammatory response was observed to correlate with an increased pro-apoptotic response and ischaemic damage in this chapter. Therefore, it is conceivable that the observed apoptotic-like cells may have represented dying monocytes/macrophages, initially recruited to remove cell debris. However, the role of inflammation in stroke, like that of apoptosis, remains a complicated and unresolved issue, where both protective and detrimental roles have been implicated.

Nonetheless, these studies support a role for apoptosis, specifically Bcl-2 family proteins Bax, Bad and Nix as well as the novel Nix-like protein in the pathophysiology of stroke. Since increased ischaemic duration was associated with a progressively increased pro-apoptotic Bcl-2 family protein response, caspase-3 cleavage, pro-inflammatory response and lesion expansion, irrespective of the cell type expressing these changes, these data support an active role for Bax, Bad, Nix and the Nix-like protein in the pathophysiology of focal cerebral ischaemia. In addition, comparison of these studies with previous *in vitro* studies (*see Chapters 3, -4 and -5*) identified similar *in vitro* and *in vivo* mechanisms of action for each of the proteins investigated. Furthermore, subcellular fractionation not only revealed that increased ischaemic duration resulted in mitochondrial accumulation of these proteins, but also identified Akt translocation towards the mitochondria as a novel intrinsic survival mechanism during permanent focal cerebral ischaemia in rats. Subsequent studies (*Chapter 7*) were designed to investigate Bax, Bad, Nix and the 30 kDa Nix-like protein responses to increased reperfusion duration in a model of transient focal cerebral ischaemia.

CHAPTER 7: TRANSIENT FOCAL ISCHAEMIA

INTRODUCTION

Chapter 6 characterised Bax, Bad, Nix and the novel 30 kDa Nix-like protein responses to permanent focal cerebral ischaemia in the rat. Using subcellular fractionation methods, mitochondrial accumulation was identified as a pivotal event in the signal transduction pathway of each protein's pro-apoptotic response. Perhaps surprisingly, a progressive increase in pro-apoptotic response was observed for each protein with increased ischaemic duration. Furthermore, the greatest magnitude of response was observed in the core striatal lesion after 24 h permanent ischaemia. Therefore, this chapter was designed to investigate the effects of increased reperfusion duration on Bcl-2 family protein activity. Reversal of the vessel occlusion (re-canalisation or reperfusion) can occur in humans, either as a result of spontaneous re-canalisation or following treatment with thrombolytic agents. Clinical (ECASS Study Group 1995, Hacke *et al.* 1999, Marchal *et al.* 1999) and animal studies (Chopp 1999, del Zoppo *et al.* 2000, Schellinger *et al.* 2003, Warlow *et al.* 2003) have shown that early restoration of blood flow salvages ischaemic brain tissue. However, re-canalisation is a complex and controversial issue with regards stroke outcome. Delayed reperfusion, while reported to reintroduce essential high-energy substrates, might exacerbate damage in already compromised tissue (Marchal *et al.* 1999, Park & Lucchesi 1999, Ghoneim *et al.* 2003). Infiltration of pro-inflammatory cytokines (Zhang *et al.* 1994, Hallenbeck *et al.* 1996, Iadecola *et al.* 1996, Kuroda & Siesjö 1997), blood brain barrier disruption and infiltration of matrix metalloproteinases (Aoki *et al.* 2002) and increased reactive oxygen species, secondary to mitochondrial dysfunction (Siesjö *et al.* 1981, Kontos *et al.* 1985, Siesjö *et al.* 1989) are proposed mediators of reperfusion damage. For example, transgenic and knockout superoxide dismutase models demonstrated protection and exacerbation of ischaemic damage in a model of transient ischaemia (Fujimura *et al.* 1999). Reperfusion injury has been well documented in other organs (Sahach *et al.* 2002, Fiorillo *et al.* 2003), and following global ischaemia (Caldwell *et al.* 1994, Cowley *et al.* 1996). However, whether such a phenomenon occurs during focal cerebral ischaemia has been debated.

The concept of cellular energy failure is central in ischaemic research. It is likely that cell necrosis is secondary to mitochondrial dysfunction and a perturbed energy metabolism (Siesjö *et al.* 1984, Siesjö *et al.* 1988). Although reperfusion has been associated with an initial restoration of mitochondrial function, mitochondrial dysfunction ensues with longer periods of reperfusion (Sims & Pulsinelli 1987, Nakai *et al.* 1997, Anderson & Sims 1999). In contrast, conflicting data exists regarding ATP levels in the ischaemic brain. Some studies have reported an initial surge, followed by a persistent

suppression of ATP in the ischaemic hemisphere (Folbergrova *et al.* 1995); Others have demonstrated almost complete restoration of ATP levels within the penumbra, with at least partial restoration of ATP in the core lesion even after extended periods of reperfusion (Hata *et al.* 2000); While one study reported complete restoration of ATP and other energy metabolites in the ischaemic hemisphere of rats after a period of 1 h MCA occlusion (Selman *et al.* 1990). Differences in these studies might be attributed to fundamental differences in the initial occlusion period and the species used, where available evidence suggests that recovery of protein synthesis, ATP levels, and ultimately mitochondrial function during recirculation might be dependent on both ischaemic- and reperfusion duration. Nonetheless, these findings suggest that sufficient energy might be available in ischaemic tissue to enable activation of energy-consuming apoptotic cell death mechanisms. In agreement with the above proposal, a growing body of evidence suggests that apoptosis might be a more prominent feature of damage induced by transient rather than permanent ischaemia (Matsushita *et al.* 1998). However, reperfusion has also been reported to promote activation of an Akt-mediated survival mechanism (Kitagawa *et al.* 1999, Friguls *et al.* 2001), which following permanent ischaemia (*see Chapter 6*) was at least in part mediated through inhibition of the pro-apoptotic Bad response. Therefore, this chapter was designed to investigate Bax, Bad, Nix and the novel 30 kDa Nix-like protein responses to increased reperfusion duration, in a rat model of transient focal cerebral ischaemia.

AIMS

The aims of this study were to (a) characterise the spatial and temporal profiles of histological damage produced in a transient model of focal cerebral ischaemia and the effects of increased reperfusion duration on the observed damage, (b) investigate the pro-apoptotic Bcl-2 family response in this transient ischaemia model, (c) investigate putative Akt-mediated survival response in a less severe model of focal ischaemia and (d) investigate ‘reperfusion injury’ in the present model by comparing observed histological and biochemical changes with data obtained in the permanent ischaemia study (*see Chapter 6*).

MATERIALS & METHODS

The materials and methods for the histological and biochemical data presented in this chapter were described in *Chapters 2 and -6*. However, methods specific to this chapter are detailed below.

MCA occlusion was induced for 1 h and animals were allowed to recover for 6 h or 24 h. The cortex and striatum of sham, 6 h and 24 h transiently-occluded rats were subsequently isolated to investigate histological and biochemical changes associated with increased ischaemic duration, as described in Chapter 6.

RESULTS

Histological Assessment of Damage

In the present study, 1 h transient focal cerebral ischaemia was induced by MCA occlusion, as this is considered to be the minimal ischaemic duration required to produce infarction (Selman *et al.* 1990). In sham-operated animals the monofilament was inserted into the CCA, advanced towards the ICA and removed immediately. Restoration of blood flow was observed under a microscope. Animals were allowed to recover for 5 h or 23 h, brains were post-fixed, thionin stained to score gross neuronal damage and subsequently re-stained using H&E to further characterize nuclear changes to identify the mechanism of cell death in the affected cells. One-hour MCA occlusion followed by 5 h reperfusion produced a lesion that was restricted to the striatum, as assessed using thionin staining. Pallor observed in the striatum following 6h transient ischaemia (*Figure 7.1*) was less obvious than the well-demarcated striatal lesion observed following 6 h permanent ischaemia (*see Chapter 6*).

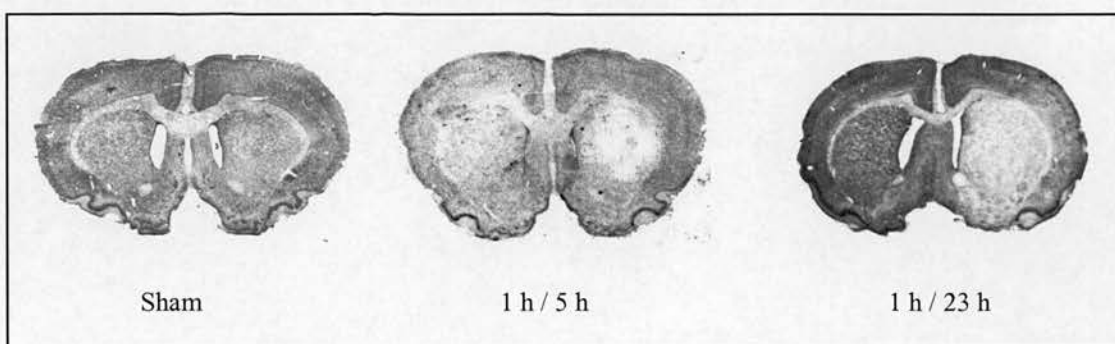


Figure 7.1: Thionin stained brain sections following transient MCA occlusion in rats.

Histological assessment of damage was performed on thionin stained sections 6 h and 24 h after onset of 1 h MCA occlusion in rats (n=3). The lesion, area of pallor, was restricted to the striatum after 6 h transient ischaemia. Increased ischaemic duration produced a lesion with both a cortical and striatal component. Sections from individual brains per treatment group.

Closer examination identified a striatal lesion with discrete, selective neuronal damage to individual cells in the deeper layers of the ipsilateral cortex. In contrast to permanent ischaemia, many neurons

within the striatal lesion appeared to be dying, rather than dead after 6 h transient ischaemia. Although many cells within the core lesion appeared swollen with vacuolisation of the cytosol, with typical necrotic-like morphological features, occasional neurones at the periphery of the striatal lesion, and isolated cortical neurones appeared shrunken with condensed, pyknotic nuclei, indicative of apoptosis. In addition, many neurones within the core lesion appeared to maintain their structural integrity, yet displayed loss of thionin staining. Since thionin stains mRNA it might be suggested that protein synthesis was reduced in those striatal and deeper cortical neurones. Cell death in these cells was likely only delayed, and not prevented. In contrast, most cortical neurones, but also some striatal neurones, appeared undamaged with large round nuclei and prominent nucleoli. Sections were subsequently re-stained using H&E to provide a more detailed account of nuclear changes in the compromised cells. Mainly necrotic features of cell death were observed in the core striatal lesion 6 h after onset of transient ischaemia (*Figure 7.2*). Few cells in the lateral striatum and deeper cortical layers, displayed pyknotic nuclei (condensed chromatin) observed as small rounded masses around the nuclear membrane. Unlike 6 h permanent ischaemia (*see Chapter 6*), the formation of apoptotic bodies around the cell membrane was not observed following 6 h transient ischaemia. These findings suggested that in addition to an early necrotic response in the core striatal lesion, with only few cells displaying features of apoptosis, good overall preservation was observed in the cortex, and less so, striatum at this early time point.

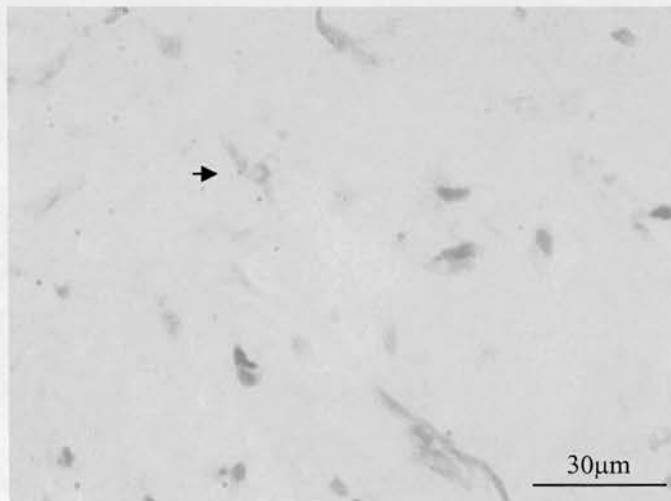


Figure 7.2: H&E staining of the ischaemic lesion after 6 h transient ischaemia.

Thionin stained sections (*see Figure 8.1*) were subsequently re-stained with haematoxylin and eosin (H&E) to characterise putative nuclear changes in dying cells. Most cells within the core striatal lesion displayed 'ghost-like' characteristics (arrow), indicative of an early necrotic response.

Volume of damage was assessed by loss of thionin staining, identified as an area of well demarcated tissue where the effects of transient ischaemia was compared against the volume of damage produced following permanent ischaemia. No significant difference was observed between the volume of striatal damage observed 6 h after onset of transient ($18.27 \pm 5.32 \text{ mm}^3$) and permanent ischaemia

($19.21 \pm 5.25 \text{ mm}^3$). At this time point, no cortical damage was observed in either treatment group (Figure 7.3). Similar volumes of damage were observed in the striatum following 24 h transient ($24.20 \pm 3.51 \text{ mm}^3$) and permanent ($20.56 \pm 4.67 \text{ mm}^3$) ischaemia. In contrast, although 24 h transient ischaemia produced a cortical lesion ($90.59 \pm 9.23 \text{ mm}^3$) this appeared to be smaller than observed after 24 h permanent ischaemia ($125.46 \pm 5.25 \text{ mm}^3$, Figure 7.4).

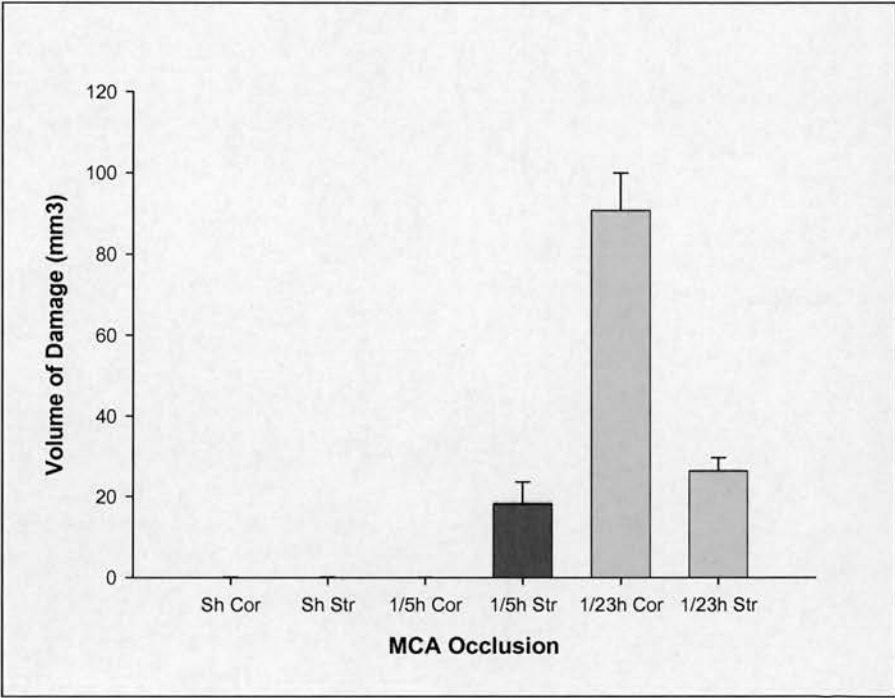


Figure 7.3: Volume of tissue damage observed with increased reperfusion duration.

Thionin stained coronal brain sections ($20\mu\text{m}$) were assessed after 6 h and 24 h transient ischaemia ($n=3$). Volume of damage, measured as area of pallor within the lesion boundary was $18.27 \pm 5.32 \text{ mm}^3$ in the striatum after 6 h and expanded into the cortex ($90.59 \pm 9.23 \text{ mm}^3$) after 24 h transient ischaemia.

Since lesion development appeared to progress at a slower rate following transient compared to permanent ischaemia, it was proposed that different cell death mechanism might underlie the apparent differences in lesion patterns in these two models of focal ischemia. H&E stained sections ($20\mu\text{m}$) from each time point were analysed as a means of providing an insight into the underlying cell death mechanisms occurring in each experimental model. Cell damage observed following 6 and 24 h transient ischaemia was similar to damage observed following treatment with neuroprotectants (Ebisu *et al.* 2001, Furuichi *et al.* 2003). Islands of cells with preserved cell structure, but likely dying more slowly than the core lesion, were observed primarily in the cortex at this time point. H&E staining revealed increased necrotic and apoptotic responses with increased reperfusion duration. An increased number of cells, primarily within the core striatal and deeper cortical regions, displayed characteristic

such as neuronal shrinkage, nuclear condensation but not fragmented apoptotic bodies following 23 h reperfusion (Figure 7.5).

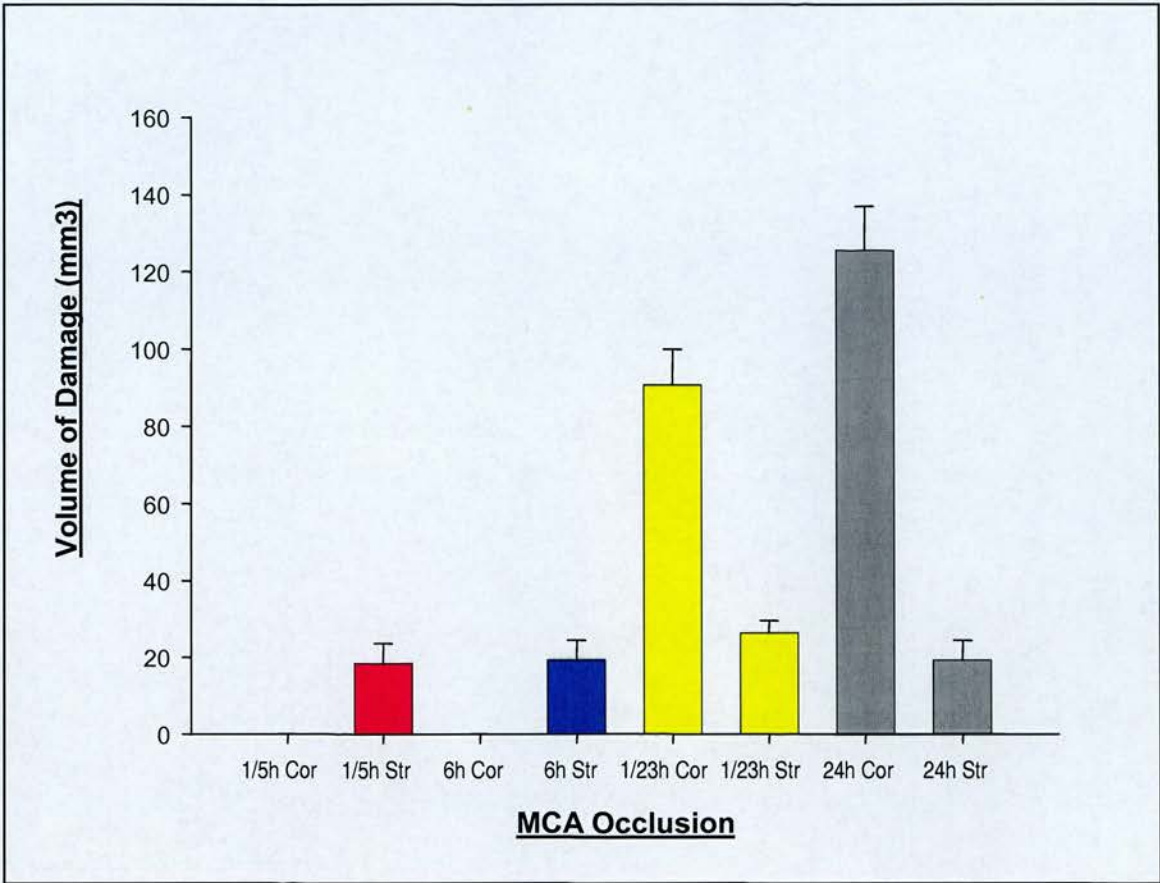


Figure 7.4: Comparison of tissue damage observed after permanent and transient cerebral ischaemia. Cortical (Cor) and striatal (Str) lesion volumes after 6 h transient and permanent ischaemia were comparable. However, 24 h transient ischaemia produced a smaller cortical lesion than 24 h permanent ischaemia, after 24 h (3 animals per treatment group).

Transient (1 h) ischaemia produced a well demarcated lesion that was restricted to the striatum after 6 h and encompassed the cortex and striatum after 24 h using thionin staining (Figure 7.1). However, H&E staining of the same sections revealed numerous islands of cells with good structural preservation, primarily in the less severely damaged cortex (Figure 7.5). Numerous shrunken, apoptotic-like cells with pyknotic nuclei were detected. Unlike 24 h permanent ischaemia (Chapter 6), however, which reported apoptotic bodies (a late stage of apoptosis), no such changes were observed following transient ischaemia. Increased detection of these apoptotic-like cells occurred with increased reperfusion duration, and primarily in the core striatal lesion. Few apoptotic cells could be detected in the deeper cortex after 6 h, supporting a role for apoptosis in lesion expansion (Figure 7.5).

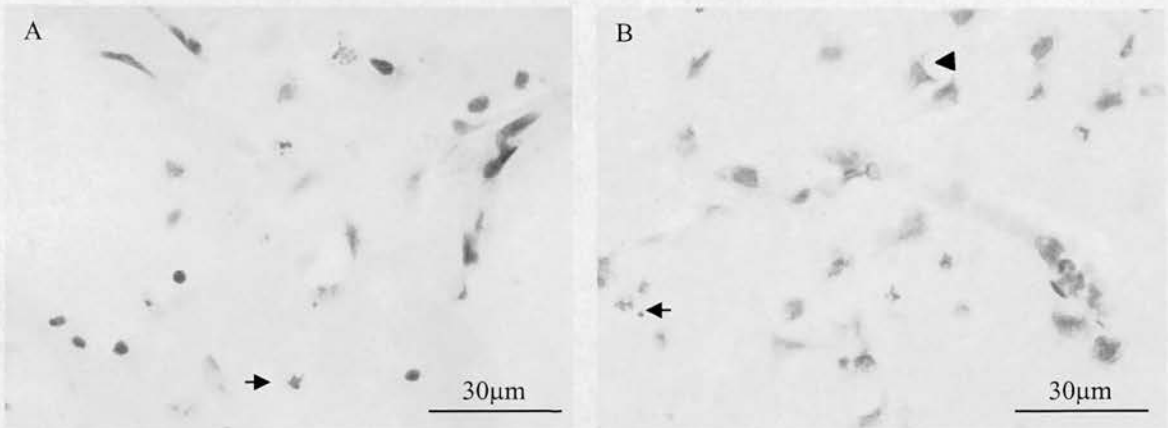


Figure 7.5: H&E staining of the ischaemic lesion after 24 h transient ischaemia.

H&E staining of the same brain sections revealed good overall cell preservation and many neurones (arrowhead) within the cortex after 24 ischaemia. Many cells were detected within the core striatal lesion (A) and the deeper cortex (B) displayed morphological features of apoptosis; cell shrinkage, pyknotic nuclei (arrow). No apoptotic bodies, indicative of end stage apoptosis were detected.

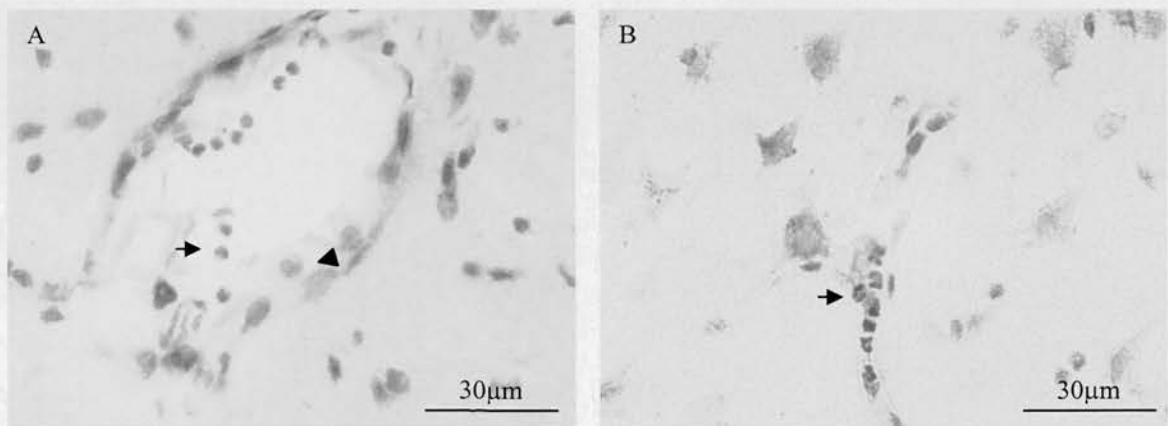


Figure 7.6: Perivascular response in the striatum and deeper cortex after 24 h transient ischaemia.

H&E stained brain sections (see Figure 8.5) subsequently identified an infiltration of small rounded, agranular cells, reminiscent of monocytes (arrow) was detected within and escaping from blood vessels primarily in the core striatal lesion (A) and also the deeper cortex 24 h after onset of transient ischaemia. Large foamy cells, resembling macrophages were observed in the striatum after 24 ischaemia (arrowhead). A second cell-type which was restricted to the blood vessels was observed within the deeper cortex (B). These cells were multi-lobed and resembled neutrophils (arrow).

A strong perivascular response was also observed in the core striatal lesion following 23 h reperfusion (Figure 7.6A) and in the deeper cortex (Figure 7.6B). Small rounded agranular cells, resembling monocytes, were observed in both the cortex and striatum whereas comparatively larger foamy cells, indicative of macrophages, were observed in the core lesion at this time point. As a monocyte or upon

leaving the blood stream to become a macrophage, these cells are phagocytic and might facilitate removal of dead cells within these damaged areas.

Biochemical Assessment of Damage

Changes in total protein expression and subcellular distribution were used to assess pro-apoptotic Bcl-2 family protein expression following transient ischaemia and to compare the observed response with the pro-apoptotic response observed following permanent ischaemia in Chapter 6. Increased Bax protein expression was observed 6 h and 24 h after ischaemic onset in the core striatal lesion. In contrast, Bax expression did not change in the ipsilateral cortex at either time point (*Figure 7.7*). Similarly, upregulation of Nix was observed in the striatum after 6 and 24 h ischaemia and less so in the cortex 24 h after onset of transient ischaemia. Upregulation of the 30 kDa Nix-like immunoreactive protein was also observed in the striatum after 6 h and 24 h ischaemia (*Figure 7.9*).

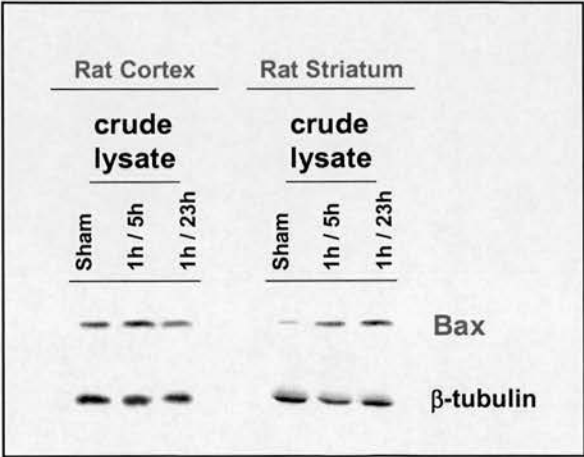


Figure 7.7: Changes in Bax protein expression after 6 h and 24 h transient ischaemia.
Total cell lysates were obtained following sham, 6 h and 24 h after onset of transient (1 h) ischaemia and analysed for changes in pro-apoptotic Bax activity (n=7). A progressive increase in levels of Bax immunoreactivity was observed with increased reperfusion duration (5-23 h) in the striatum. No changes were observed in the cortex at any time point. Equal loading was confirmed using β-tubulin.

Translocation from the cytosol to the mitochondria was not observed in any brain region, or at any time point investigated for Bax (*Figure 7.8*). In contrast, an early reduction in the 30 kDa Nix-like protein immunoreactivity was observed in both cell fractions of the striatum after 5 h reperfusion. Increased reperfusion was associated with mitochondrial accumulation of Nix to pre-ischaemic levels (*Figure 7.10*). Upregulation of Nix was observed in the cytosolic fraction of the striatum after 6 h, with concomitant mitochondrial accumulation of Nix 24 h after onset of ischaemia (*Figure 7.7*).

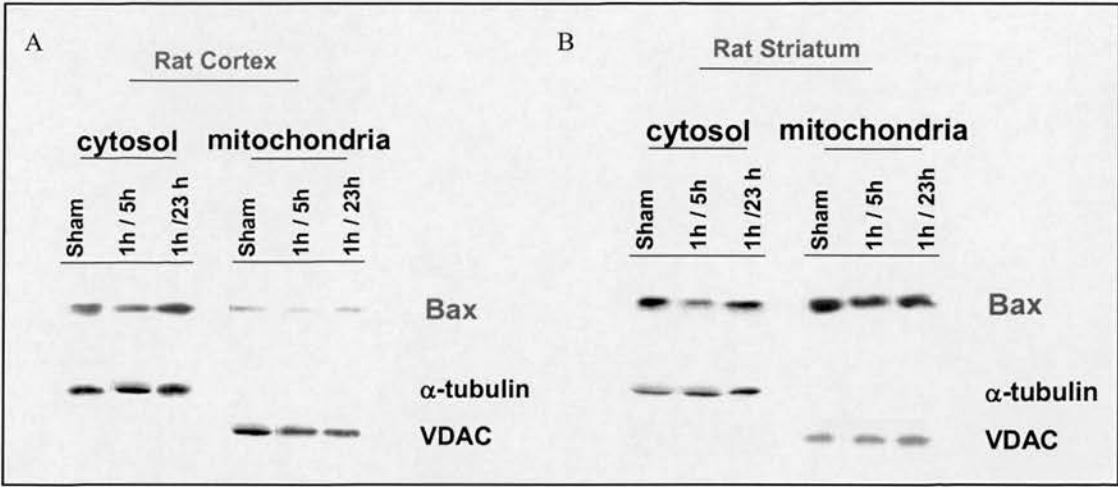


Figure 7.8: Changes in the subcellular distribution of Bax following transient ischaemia.

Lysates were subsequently separated into cytosolic and mitochondrial fractions and assessed for putative changes in the subcellular distribution of Bax (n=7). As previously observed (see Chapters 3 and -7), Bax appeared as a primarily cytosolic and less so mitochondrial-associated protein in sham animals. No obvious changes were detected in the subcellular distribution of Bax in either the cortex (A) or striatum (B) following 5 h or 23 h reperfusion. Fraction purity and equal loading was confirmed using cytosolic and mitochondrial protein markers, α -tubulin and VDAC, respectively.

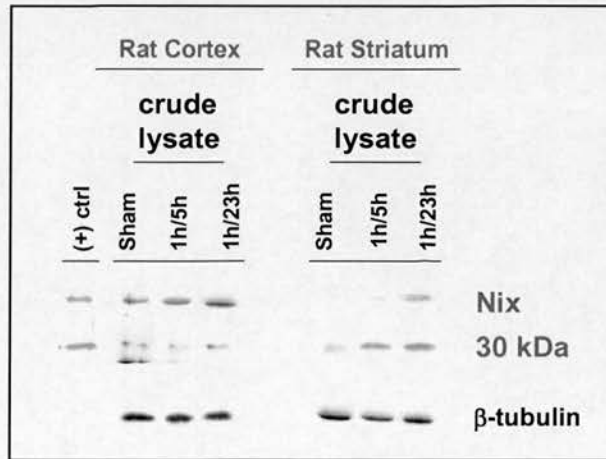


Figure 7.9: Changes in Nix protein expression after 6 h and 24 h transient ischaemia.

Total cell lysates were obtained following sham, 6 h and 24 h after onset of transient (1 h) ischaemia and analysed for changes in pro-apoptotic Nix activity (n=7). A progressive increase in Nix immunoreactivity was observed in the striatum with increased reperfusion duration (5-23 h). Slight induction of Nix was observed in the cortex after 24 h. Levels of the 30 kDa Nix-like protein expression increased in the striatum at both time points, but were unchanged in the cortex. β -tubulin confirmed equal loading.

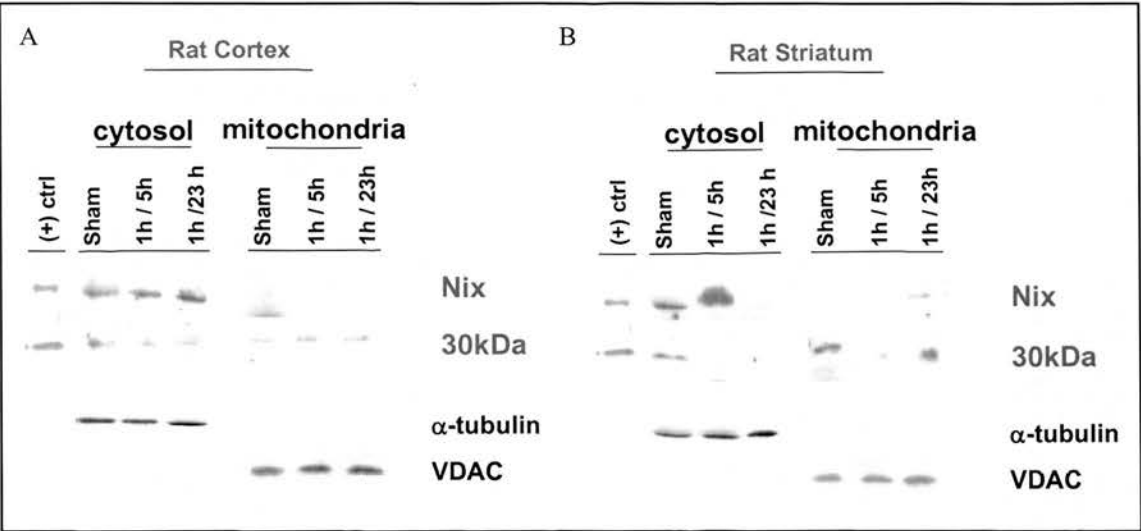


Figure 7.10: Changes in the subcellular distribution of Nix following transient ischaemia.

Cytosolic and mitochondrial fractions were assessed for changes in the subcellular distribution of Nix and the 30 kDa Nix-like protein (n=7). Nix was detected in the cytosolic fraction of cortex (A) and striatum (B) of sham animals. *De novo* synthesis Nix was detected in the cytosol of the striatum after 6 h ischaemia, with translocation towards the mitochondria after 24 h transient (1 h) ischaemia. Upregulation of cytosolic Nix, but without mitochondrial accumulation, was observed in the cortex at this time. In contrast, the 30 kDa Nix-like protein was detected in both the cytosol and mitochondria of sham animals. Downregulation of the Nix-like protein was observed in both cell fractions of the striatum after 6 h ischaemia, with mitochondrial accumulation and restoration of this protein to pre-ischaemic values after 24 h ischaemia. No significant change in Nix expression was observed in the cortex. VDAC and α -tubulin confirmed fraction purity.

Total Bad expression did not change in either brain region in response to 6 h transient ischaemia (Figure 7.11). In contrast, a slight reduction in P¹³⁶ Bad immunoreactivity was detected in the cortex and striatum at this point, where a cytosolic reduction of P¹³⁶ Bad was revealed using subcellular fractionation methods (Figure 7.12). However, a concomitant mitochondrial accumulation of total Bad was not detected, or was below levels of detection.

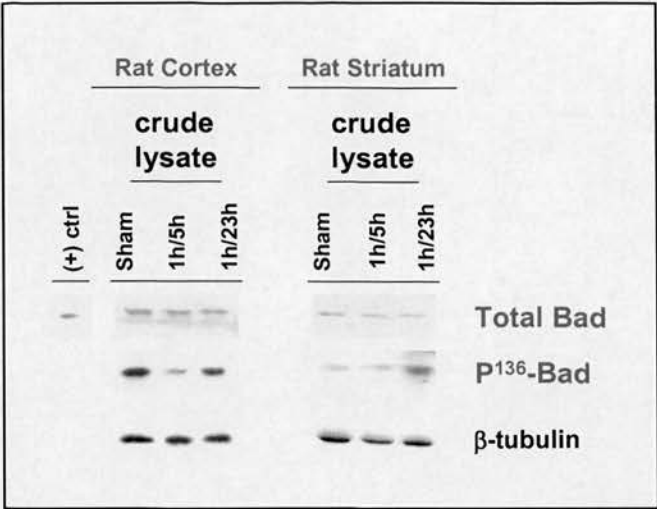


Figure 7.11: Bad protein expression and phosphorylation status after transient ischaemia.

Total cell lysates were obtained 6 h and 24 h after onset of transient (1 h) ischaemia in rats and analysed for changes in pro-apoptotic Bad activity (n=7). P¹³⁶ Bad immunoreactivity decreased in the cortex after 6 h transient ischaemia. Total Bad expression did not appear to change at this time point. Restoration of cortical P¹³⁶ Bad expression to pre-ischaemic values and increased P¹³⁶ Bad expression in the striatum was detected with increased reperfusion duration (23 h). B-tubulin confirmed equal loading.

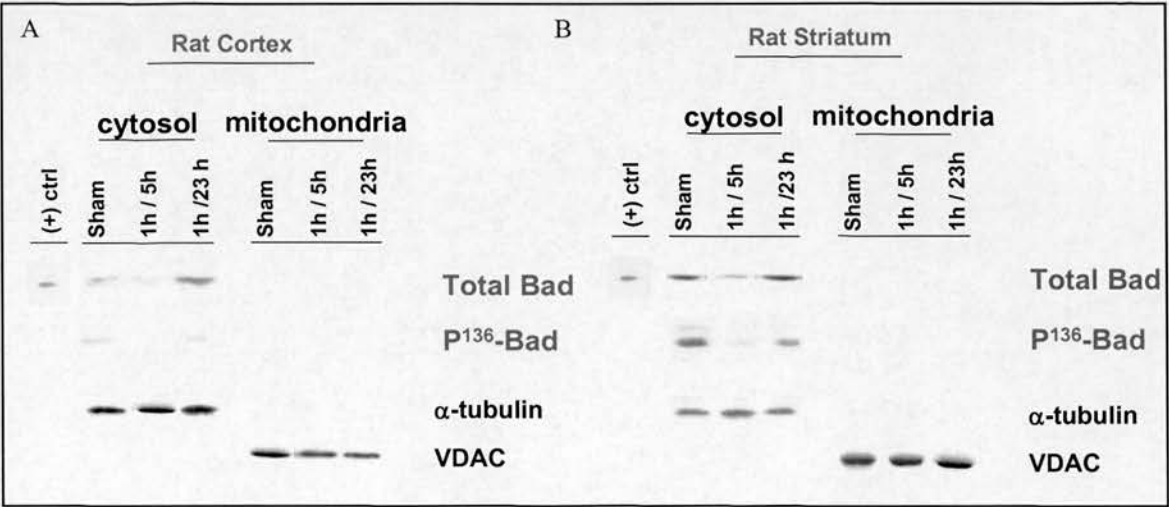


Figure 7.12: Subcellular distribution of Bad and P¹³⁶Bad 6 h and 24 h after transient ischaemia.

Lysates were separated into cytosolic and mitochondrial fractions and assessed for changes in the subcellular distribution of total and P¹³⁶ Bad (n=7). Bad appeared cytosolic and phosphorylated in sham animals. A reduction in total and P¹³⁶ Bad immunoreactivity was observed in the cytosol of the cortex and striatum with prolonged reperfusion (23 h). However, a concomitant mitochondrial accumulation of dephosphorylated Bad was not observed. Antibody specificity was confirmed using a Bad peptide supplied with the Bad antibody. VDAC and α-tubulin confirmed fraction purity and equal loading.

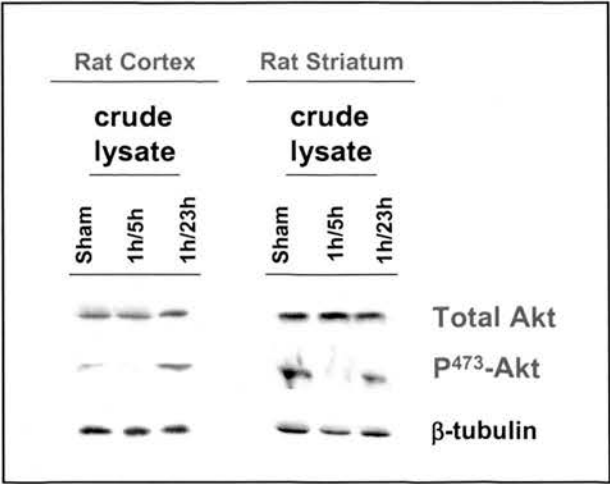


Figure 7.13: Akt and P⁴⁷³ Akt protein expression following transient focal cerebral ischaemia
 Total cell lysates were analysed for changes in Akt and P⁴⁷³ Akt immunoreactivity with increased reperfusion duration (n=7). Total Akt expression did not change at any time point investigated. In contrast, a reduction in P⁴⁷³ Akt expression was observed in the cortex and striatum after 6 h ischaemia. Increased reperfusion duration (23 h) was associated with increased levels of P⁴⁷³ Akt in the cortex and return to pre-ischaemic values in the core striatal lesion. B-tubulin confirmed equal loading.

While total Akt levels did not change in response to 6 h transient ischaemia, a reduction in P⁴⁷³ Akt was observed in both the cortex and striatum at this time point (Figure 7.13). The subcellular distribution of P⁴⁷³ Akt did not appear to change after 6 h ischaemia (Figure 7.14). In contrast, restoration of total Akt to pre-ischaemic values in the striatum, and an increased total Akt expression was observed in the cortex with prolonged reperfusion duration (23 h) (Figure 7.13). In contrast, subcellular fractionation studies demonstrated mitochondrial accumulation of Nix after 24 h ischaemia (Figure 7.14) and further support the use of subcellular fractionation as a more accurate tool to measure protein activity.

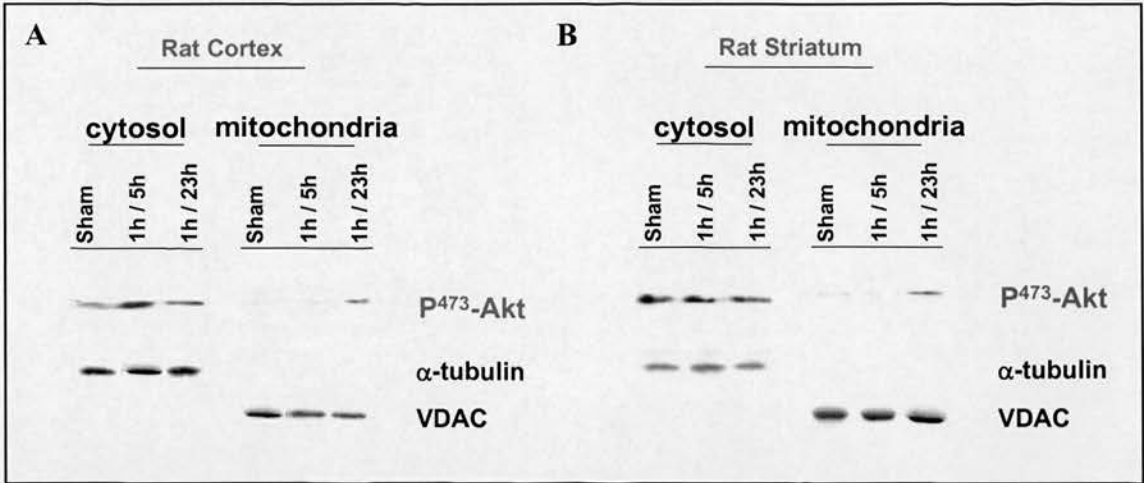


Figure 7.14: Assessment of P^{473} Akt subcellular distribution after 6 h and 24 h transient ischaemia.

Lysates were separated into cytosolic and mitochondrial fractions and assessed for changes in the subcellular distribution of P^{473} Akt (n=7). P^{473} Akt was observed as a primarily cytosolic, but also mitochondrial-associated protein in sham animals. P^{473} Akt immunoreactivity did not change in either brain region after 6 h of transient (1 h) ischaemia. In contrast, mitochondrial accumulation of P^{473} Akt was observed in both the cortex (A) and striatum (B) with increased reperfusion duration (23 h). However, a concomitant decrease in cytosolic P^{473} Akt was not detected. VDAC and α -tubulin confirmed fraction purity.

In the previous chapter, permanent ischaemia was associated with a progressive increase in cleaved caspase-3, which was used as a marker of apoptotic cell death (see Chapter 6). In the present study, increased levels of cleaved caspase-3 were also observed to correlate with increased ischaemic damage. Cleaved caspase-3 was only observed in the cytosolic fraction of the core striatal lesion after 24 h transient ischaemia (Figure 7.15), further supporting a role for apoptosis in lesion expansion.

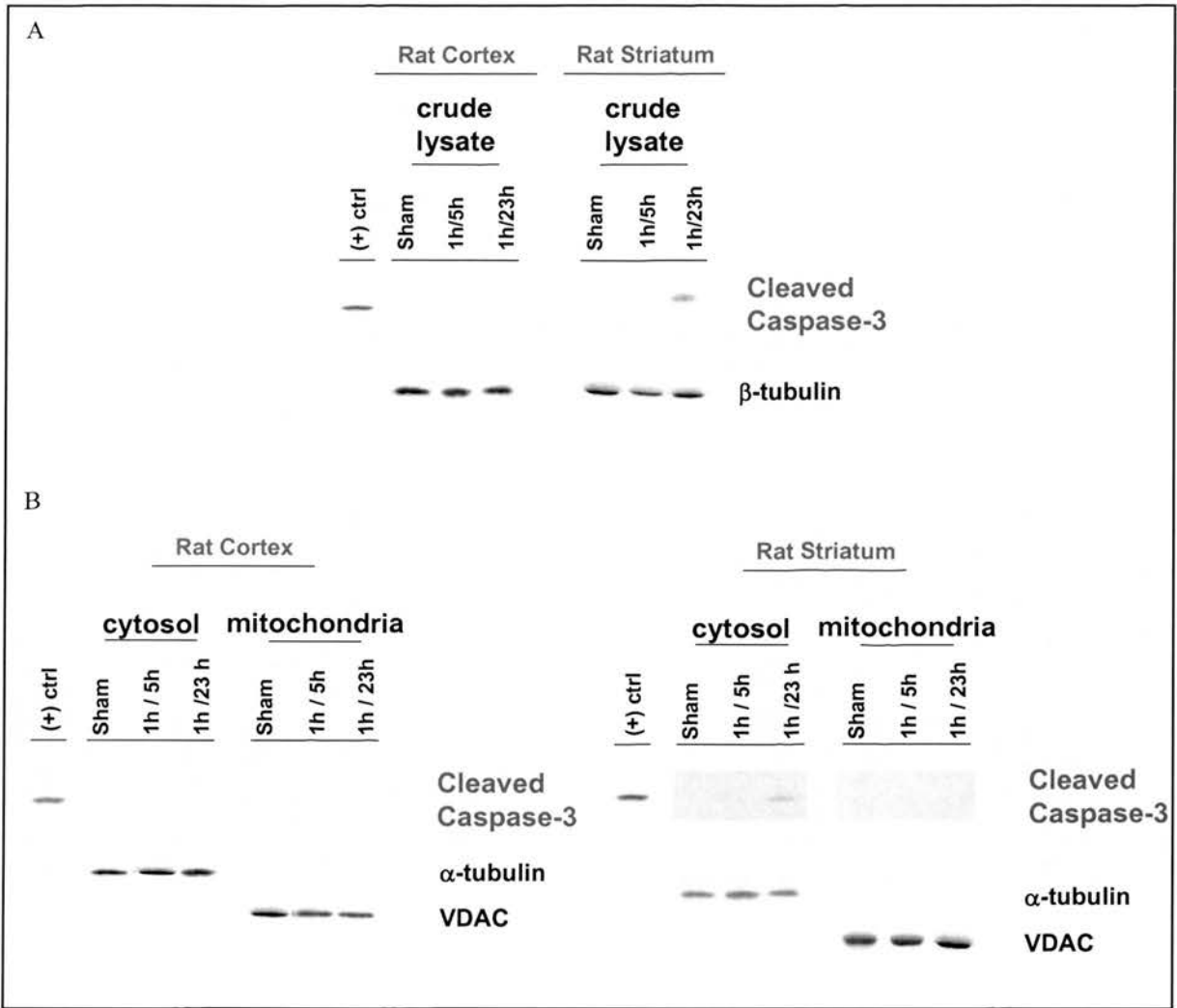


Figure 7.15: Cleaved caspase-3 expression and subcellular distribution after transient ischaemia.

Total cell lysates, cytosolic and mitochondrial fractions were analysed for changes in cleaved caspase-3 protein expression and subcellular distribution after 6 h and 24 h transient ischaemia (n=7). Cleaved caspase-3 expression was only detected after 24 h in the core striatal lesion (A) and appeared to be restricted to the cytosolic fraction (B). Apoptotic rat cerebellar granule cells (*see Chapter 4*) confirmed antibody specificity. β -tubulin, VDAC and α -tubulin confirmed equal loading in whole cell lysates, mitochondria and cytosolic fractions, respectively.

DISCUSSION

Coronal brain sections (20µm) from sham, 6 h and 24 h transiently-occluded rats were initially stained with thionin to evaluate the effects of reperfusion on gross histological features of cell death and the volume of damage. Sections were subsequently re-stained with H&E to investigate specific nuclear changes and to provide a more detailed insight into the mechanism of cell death. A lesion restricted to the striatum at 6 h, was observed to expand into the cortex after 24 h transient ischaemia. Although the pattern of damage was comparable in both permanent and transient models of ischaemia, the volume of damage appeared to differ slightly. The volume of damage after 6 h permanent (*see Chapter 6*) and transient ischaemia was comparable, however, a smaller lesion with less cortical damage was observed after 24 h transient compared to permanent ischaemia. Also, cells in the ischaemic hemisphere of transiently occluded animals were of a higher density than observed after permanent MCA occlusion. Histological data suggested that, after 1 h MCA occlusion in rats, increased reperfusion duration promoted lesion expansion. However, in comparison to permanent ischaemia (*Chapter 6*), a comparatively delayed progression of cell death was observed following transient focal cerebral ischaemia. Reperfusion, if initiated within 3 h in humans (Hacke *et al.* 1999) and 90-120 min in rats (Kaplan *et al.* 1991, Memezawa *et al.* 1992b) has been reported to produce smaller infarcts. In agreement, a comparatively smaller lesion volume was observed after 24 transient compared to permanent ischaemia (*Chapter 6*). In contrast, some authors have argued that although the lesion might appear initially smaller at earlier time points, reperfusion might only delay progression of the ischaemic damage, since the final lesion volume after infinite time has been reported to be comparable to a mature lesion produced following permanent vessel occlusion (Chopp *et al.* 1996, Phillis *et al.* 1997). Although lesion expansion after 24 h was not investigated in the present model of transient ischaemia, the morphological appearance of 'dying' rather than 'dead' cells at this time point might support a model where reperfusion merely delays ischaemic damage and warrants further investigation.

Traditionally, cell death has been considered necrotic in the core ischaemic lesion on the basis of location, time elapsed from the insult, loss of basophilia, and the presence of karyorrhexis. However, the main characteristics of necrosis, cell and organelle swelling and rupture (Wyllie *et al.* 1980), are rarely observed in neurones within the core lesion. Instead, swollen morphology is often associated with apoptotic features, such as pyknotic nuclei, and cytoplasmic vacuolation (Ferrer *et al.* 2003) as was reported in this chapter. An acute necrotic lesion was observed in the striatum after 6 h, with detection of only a few shrunken apoptotic-like cells in the deeper cortex (Kerr *et al.* 1972). Lesion expansion into the cortex after 24 h ischaemia was accompanied by a marked increase in detection of apoptotic-like cells in the core striatal lesion and deeper cortex. However, in contrast to 24 h permanent ischaemia (*Chapter 6*), apoptotic bodies were not detected in the core lesion after 24 h

transient ischaemia, supporting a model where reperfusion delays ischaemic damage. These findings are comparable to a similar study where a mixed apoptotic/necrotic morphology was reported in the cortex and striatum. Interestingly, lesion expansion into the cortex was reported after 7 h transient (1 h) ischaemia (Charriaut-Marlangue *et al.* 1996). Whether 6 h reperfusion in the model described in this thesis would have produced a similar pattern of cortical and striatal damage is unknown. Further supporting data in this chapter, DNA fragmentation has been reported localised to the inner boundary of the core lesion using comparable models of ischaemia (Gavrieli *et al.* 1992, Charriaut-Marlangue *et al.* 1996). Also, the greatest magnitude of apoptotic response was reported in the most severe experimental paradigm (Li *et al.* 1995) further supporting a role for apoptotic cell death in ischaemic cell death.

An early pro-apoptotic response was observed for Bax, Bad, Nix and the 30 kDa Nix-like immunoreactive protein following 5 h reperfusion. Upregulation of Bax, Nix and the 30-kDa protein was observed in the core lesion, but not cortex, after 6 h transient ischaemia, whereas dephosphorylation of Bad was detected in both brain regions. These findings are consistent with histological damage that appeared to be restricted to the striatum at this time point. A progressive increase in Bax, Nix and the 30 kDa protein expression was observed in the striatum with increased reperfusion duration. Upregulation of Nix was also observed in the cortex after 24 h transient ischaemia. However, in contrast to 24 h permanent ischaemia which was associated with translocation of each protein to the mitochondria, only slight mitochondrial accumulation was not observed following transient ischaemia in the striatum. This might suggest that while increased reperfusion duration was associated with progressively increased pro-apoptotic Bax, Nix and 30 kDa Nix-like responses, and that these changes correlated with lesion expansion, the pro-apoptotic response appeared sub-maximal, without complete translocation, and in an almost delayed-like fashion compared to permanent ischaemia. The delayed appearance of apoptotic-like morphological changes following 24 h transient compared to permanent ischaemia (apoptotic bodies-late stage of apoptosis—only detected in latter model), supports the hypothesis that reperfusion delays histological and biochemical manifestations of ischaemic damage. These findings are in agreement with other transient ischaemia studies that reported a delayed cell death (Du *et al.* 1996, Prakasa *et al.* 2000) and demonstrated that dying neurones were shown to exhibit a high Bax immunoreactivity (Krajewski *et al.* 1999). One study correlated a progressive increase in Bax and cytochrome c immunoreactivity with TUNEL positive staining between 6 h and 24 h after 1 h transient occlusion in rats. However, these pro-apoptotic changes were reported primarily in the penumbra (Prakasa *et al.* 2000). After 24 h ischaemia and in agreement with data presented in this chapter, lethal changes were observed, such as neuronal shrinkage, eosinophilic cytoplasm, nuclear condensation and fragmented apoptotic bodies (Prakasa *et al.* 2000). One major discrepancy between that study and data presented in this chapter was that Prakasa *et al.* observed a cortical and striatal lesion after 6 h, compared to a striatal lesion in

this chapter; therefore it might not be surprising that apoptotic changes were comparatively less obvious in this chapter.

Upregulation and translocation of Bax has been reported after 6 h transient (30 min MCA occlusion) ischaemia in the rat (Cao *et al.* 2001). These findings support data presented in this chapter where a slight induction of Bax expression was observed in the striatum after 6 h ischaemia. However, unlike the study by Cao and colleagues, mitochondrial accumulation of Bax was not observed in this chapter. Interestingly, Cao *et al.* (2001) reported Bax changes which appeared to occur before concomitant changes in cleaved caspase-3 expression. Increased levels of cleaved caspase-3 immunoreactivity were also observed in the studies reported in this chapter, but only in the striatum after 24 h ischaemia. Nevertheless, these findings support a role for apoptosis in the progression of ischaemic cell damage.

An almost immediate and complete restoration of blood flow, primarily in the cortex, was recently reported in a similar model of transient MCA occlusion (Aoki *et al.* 2002). The release of caspase-activating factors such as cytochrome c and procaspase-9 from mitochondria of ischaemic neurones also provides strong evidence supporting a role for mitochondria-mediated energy-consuming cell death mechanisms during transient focal cerebral ischaemia (Fujimura *et al.* 1998, Krajewski *et al.* 1999, Cao *et al.* 2001). Release of another intermembrane protein, Smac/DIABLO has also been recently reported early during focal cerebral ischaemia (Shibata *et al.* 2002). Although this event was proposed to be non-specific and rather occur as a result of rapid metabolic and mitochondrial stress (Cao *et al.* 2001) and therefore interpretation of data reporting release of mitochondrial intermembrane proteins, such as cytochrome c, should be treated with caution.

Although an early pro-apoptotic response was observed for each Bcl-2 family member investigated neither concomitant cytochrome c release nor caspase-9 activation was investigated. Attempts to measure such changes were prevented due to lack of antibody specificity. An early and progressively increased response was observed for Bax, Nix and the 30 kDa Nix-like protein, however mitochondrial accumulation was only observed for Nix in the core striatal lesion after 24 h ischaemia. Therefore, it might be proposed, that although an increased pro-apoptotic response was observed with increased reperfusion duration and in the most severely damaged tissue, the ischaemic insults in this study were insufficient to promote complete translocation of these proteins. This might explain why only minimal caspase-3 immunoreactivity was observed in the core striatal lesion after 24 h transient ischaemia, compared to the pro-apoptotic response observed in the core after only 6 h permanent ischaemia (*see Chapter 6*). The above findings suggest that while 24 h transient ischaemia appeared to promote a pro-apoptotic response of some (Bax, Nix and 30 kDa Nix-like protein) Bcl-2 family members, 24 h transient ischaemia in the present study might be considered a 'less severe' insult than 24 h permanent ischaemia, since translocation and concomitant mitochondrial accumulation of Bax,

Nix and the 30 kDa Nix-like protein was observed in the core lesion after 24 h permanent ischaemia (see Chapter 6). This proposed mechanism cannot be reconciled with lack of available energy since the greatest magnitude of pro-apoptotic response was observed after 24 h permanent ischaemia, perhaps the most severe model of focal cerebral ischaemia in this thesis. Interestingly, the greatest magnitude of pro-apoptotic response in this model of transient ischaemia was also observed in the core striatal lesion after 24 h ischaemia.

Interestingly, upregulation of Bax, Nix and the Nix-like protein were observed in the striatum after 6 h transient ischaemia, which correlated with histological damage. These findings strongly suggest that initiation of an apoptotic response, mediated through the Bcl-2 family, is an early response following an ischaemic insult, and might mediate lesion expansion into the cortex observed in both transient and permanent ischaemia. Increased reperfusion duration was associated with expansion of the lesion into the cortex with a concomitant increase in pro-apoptotic Bcl-2 family response (slight upregulation of Bax and Nix) in the cortex. However, cell damage and the pro-apoptotic response in the transient model appear to progress in a delayed fashion when comparing findings with permanent ischaemia 6-24 h after onset of ischaemia. These findings suggest that, at least in these two ischaemia models, reperfusion appears to delay, but not prevent, the tissue response to focal cerebral ischaemia. Also, whether the observed pro-apoptotic response promoted lesion expansion or was the consequence of ensuing ischaemic damage remains unclear.

An early pro-apoptotic Bad/Akt response was observed. This was demonstrated by dephosphorylation of each protein, early during reperfusion (5 h). However, increased reperfusion duration was associated with an intrinsic survival response, as restoration of total cytosolic Bad, P¹³⁶ Bad and P⁴⁷³ Akt protein expression to pre-ischaemic values was observed in the striatum, with increased expression in the better perfused cortical tissue. In addition, similar to the novel Akt/Bad signalling response observed after 6 h permanent ischaemia, P⁴⁷³ Akt translocation towards the mitochondria was observed with concomitant phosphorylation of Bad following increased reperfusion duration. Increased Akt and P⁴⁷³ Akt expression has been reported between 2-6 h after onset of transient (1 h) ischaemia in both rats (Friguls 2001) and mice (Noshita *et al.* 2001). Akt immunoreactivity in these studies decreased after 24 h transient ischaemia, which was reported to correlate with increased caspase-3 activation. Chapter 6 investigated the effects of permanent ischaemia on Bad activity and demonstrated loss of the Akt/Bad survival response after 24 h permanent ischaemia, which was accompanied by increased levels of cleaved caspase-3 expression and apoptotic cell death. In contrast, a delayed and prominent activation of the Akt signalling pathway with concomitant inhibition of pro-apoptotic Bad activity was observed in the present study investigating 24 h transient ischaemia. Although inconsistent with other transient ischaemia studies discussed above (Friguls *et al.* 2001, Noshita *et al.* 2001), data presented in this chapter were consistent with the comparatively delayed

lesion expansion observed in this model of transient ischaemia. As previously discussed, the extent and pattern of infarction has been reported to vary slightly between research groups. Since, the aforementioned studies did not report histological damage it was difficult to reconcile the slight discrepancies in the temporal profile of the observed Akt-mediated survival response in each study. It might be suggested that in these models, 24 h transient ischaemia, particularly in the mouse, might have produced a more extensive lesion than observed in this study and might account for reduced levels of Akt after 24 h ischaemia in some studies. Simultaneous changes in P¹³⁶ Bad were not reported in those studies. Upregulation of P⁴⁷³ Akt has been reported in the ischaemic lesion 4 h after onset of transient (1 h) ischaemia in mice; an insult sufficient to produce a morphologically mature lesion encompassing the entire MCA territory (Friguls *et al.* 2001). Importantly, most P⁴⁷³ Akt was observed to co-localise with Neon, a neuronal marker, suggesting that the Akt survival mechanism observed in the present study might reflect an intrinsic neuronal survival mechanism. Interestingly, mitochondrial accumulation of P⁴⁷³ Akt was observed in both the cortex and striatum after 23 h reperfusion. These findings were supported by other studies which have reported Akt to function at both pre-mitochondrial and post-mitochondrial sites (Cardone *et al.* 1998). However the implications of such findings remain unclear.

In conclusion, these studies demonstrated that pro-apoptotic Bcl-2 family proteins respond differently to increased reperfusion duration. For example, a progressive increase in the pro-apoptotic response was observed for Bax, Nix and the Nix-like protein with increased reperfusion duration, while a dual response was reported for Bad following transient ischaemia. Although increased reperfusion duration was associated with lesion expansion, both histological and biochemical features of cell death appeared to be delayed in comparison to permanent ischaemia. These findings support a model where reperfusion delays ischaemic damage. Perhaps most intriguing was the observed Akt/Bad survival response with increased reperfusion duration. Moreover, this novel Akt-mediated signalling cascade was also reported early during permanent ischaemia (*Chapter 6*), further supporting an intrinsic survival mechanism in the ischaemic brain. Together, these findings argue against a model of reperfusion injury. Furthermore, cell death mechanisms mediated by Bax, Nix and the Nix-like protein might have been countered by activation of Akt/Bad survival response. Activation of this novel intrinsic survival mechanism might underlie and explain the absence of a cortical lesion after 6 h permanent ischaemia as well as the comparative delay in ischaemic damage observed after 24 h transient ischaemia. Should these speculations withstand experimental verification, this intrinsic novel Akt/Bad signalling cascade might provide a novel therapeutic target in stroke research.

CHAPTER 8: FINAL DISCUSSION

EXPERIMENTAL RATIONALE

Tissue damage associated with neurodegenerative diseases, such as Alzheimer's, Parkinson's, and Huntington's disease, motor neurone degeneration, multiple sclerosis, amyotrophic lateral sclerosis, and stroke involve cell death. Cell death in all tissues can occur along a continuum between two very different processes – necrosis, which has been identified and characterised for over a century, and apoptosis a process not identified until the 1960s which only in the last decade has been extensively investigated using *in vitro* techniques (Lockshin & Williams 1965). Necrosis is poorly regulated, pro-inflammatory and pathological, and it involves leaky mitochondrial and plasma membranes, rapid bioenergetic deterioration, cell swelling and lysis. Necrosis is relatively easily detected *in vivo* (Locke and Brauer 1991) and tends to be associated with an intense, acute tissue insult such as exposure to high levels of toxicants or at the centre of a local ischaemic insult. In sharp contrast, apoptosis tends to occur during less intense, chronic tissue insults, such as low-level toxicant treatment, in the perifocal regions surrounding a core necrotic focal ischaemic infarct and in most types of neurodegenerative diseases. Apoptosis is a highly regulated, non-inflammatory, ATP-consuming process that maintains plasma membrane integrity, involves nuclear fragmentation, chromatin condensation and compartmentalisation into apoptotic bodies that are phagocytosed by neighbouring cells (Brown *et al.* 1999, Kroemer and Reed 2000).

Animal models of focal cerebral ischaemia are now generally accepted as pertinent to the human condition (Gingsberg & Busto 1989, Hossmann *et al.* 1998). Permanent or transient (with reperfusion) occlusion of the middle cerebral artery (MCA) in the rat has been extensively used to investigate the pathophysiology and pharmacotherapy of cerebral ischaemia. This produces localised infarction in the vascular territory encompassing the striatum and overlying cortex. Induction of focal ischaemia results in a range of moderate to severe reductions of cerebral blood flow. A zone of dense ischaemia, designated the ischaemic core, with cerebral blood flow less than 10% of pre-occlusion levels has been reported, around which moderately severe ischaemic tissue prevails; sometimes referred to as the 'penumbra', where blood flow has been documented 30-35% of pre-ischaemic values (Hossmann *et al.* 1994). Similar values have been reported within F.I.N.E. laboratories (*personal communication with Dr. John Sharkey*). Neuronal injury and the potential for reversal are related to the duration of the initial ischaemic episode and to the degree of reduction in blood flow in the occluded artery (Hossmann *et al.* 1973, Siesjö *et al.* 1982). Damage in the perifocal tissue, but less commonly in the focal tissue, can be ameliorated by a range of pharmacological or physical interventions initiated

around the time of vessel occlusion (Dirnagl *et al.* 1990, Gill *et al.* 1992, Park & Hall 1994). Previous studies performed within F.I.N.E. laboratories demonstrated that although the final lesion volumes were indistinguishable, the magnitude of protection varied between transient and permanent MCA occlusion in rats (McCarter 2001). Therefore, apparently substantial differences might exist in their underlying mechanisms of cell death.

At present, the only accepted therapeutic strategy in the clinic involves administration of tPA, a thrombolytic agent, which is based on rapid (therapeutic window – 3 h) restoration of blood supply and nutrients to the ischaemic tissue. However, reperfusion-injury has been observed in both human (Kidwell *et al.* 2001) and animal (Manabat *et al.* 2003) stroke studies and therefore argue against the benefits of such restorative therapy. Delayed cell death, which has been observed in less severely damaged perifocal tissue, is thought to involve, at least in part, an active type of cell death (Linnik *et al.* 1993, Chopp *et al.* 1996). The relative contribution of apoptotic cell death to the observed ischaemic tissue damage is controversial and a subject of much debate. Some investigators argue against a role for apoptosis, primarily because the typical electron microscopic features are infrequently detected, except in the brains of neonatal hypoxic victims. Due to difficulties in obtaining suitable post-mortem samples at appropriate time-points (Mattson *et al.* 2000), there are no compelling published data for or against apoptosis in human stroke tissue at the present time. Neurones at the border of the ischaemic territory can however survive for hours or even days following an ischaemic insult (Dereski *et al.* 1993). Similarly, MRI studies have suggested that the progression of ischaemic damage is also delayed in stroke patients (Baird *et al.* 1997), where symptoms improved during the first week after an insult (Jorgensen 1995), reinforcing the importance of delayed mechanisms of ischaemic cell death, including apoptosis and inflammation in humans. Supporting such a proposal, numerous studies have reported DNA fragmentation, apoptotic morphology such as apoptotic bodies as well as changes in gene expression of apoptotic regulators, such as immediate early genes, heat shock proteins, caspases and the Bcl-2 family following stroke (Gillardon *et al.* 1996, Cao *et al.* 2001).

The hypothesis that mitochondria control cell death has passed through successive phases of neglect, disdain, suspicion, and (partial) acceptance (Green & Reed 1998). Stroke can be viewed as a disease of mitochondrial dysfunction (Fiskum *et al.* 2000, Sims & Anderson 2002), and, as central regulators of the mitochondrion, the Bcl-2 family could offer the possibility of modifying the outcome of this disabling disease. In support of this view, Bcl-2 over-expression resulted in a reduced infarct size (Zhao 2003). Supporting a central role for these regulatory proteins in focal cerebral ischaemia was the recent finding that over-expression, or viral delivery of anti-apoptotic Bcl-2 family proteins each reportedly conferred neuronal protection with reduced infarct sizes (Sun *et al.* 2003, Zhao *et al.* 2003). However, at the time of study few studies reported concomitant protein alterations (Gillardon 1996,

Du *et al.* 1996, Prakasa *et al.* 2000) which would more accurately reflect functional responses to stroke. Therefore, the present study was designed to investigate putative Bcl-2 family response(s) to focal cerebral ischaemia in the rat, with the aim of identifying novel pro-apoptotic Bcl-2 family members and/or confirming previously reported Bax (Gillardon *et al.* 1996) as therapeutic targets in stroke research.

Permanent and transient models of focal ischaemic stroke were set-up using the monofilament model and the technique optimized to a standard comparable with other researchers within F.I.N.E. – assessed by measuring the volume and pattern of ischaemic damage in each treatment group. Chapter 2 examined the putative involvement of the Bcl-2 family of regulatory proteins in a rat transient model of stroke. Dying neurones did not appear to display apoptotic-like morphological changes in either the ischaemic core or less damaged perifocal regions. Furthermore, initial biochemical investigations failed to detect changes in pro-apoptotic Bcl-2 family proteins, Bax, Bad and Nix or cleaved caspase-3, negating a significant role for apoptotic cell death in stroke pathology, at least in this experimental model of stroke. *In vitro* models of apoptosis were set-up and used to characterise the regulatory mechanisms of Bax, Bad and Nix, as examples of three sub-types of pro-apoptotic Bcl-2 family proteins, using a less complex experimental system (*Chapters 3, -4 and -5*). The putative roles of pro-apoptotic proteins Bax, Bad and Nix were subsequently investigated using less severe models of both permanent and transient focal cerebral ischaemia in the rat (*see Chapters 6 and -7*). Morphological and biochemical evidence was provided to support a role for apoptosis, specifically the pro-apoptotic Bcl-2 family proteins, Bax, Bad and for the first time, Nix, in the pathophysiology of focal cerebral ischaemia in the rat. Finally, the observed *in vitro* and *in vivo* mechanisms of action for each protein were compared and the less complex *in vitro* model of apoptosis was identified as a suitable model to further characterise the intracellular molecular circuitry for each of the three Bcl-2 family proteins investigated with the ultimate aim of identifying novel therapeutic targets for stroke research (*Figure 8.1*).

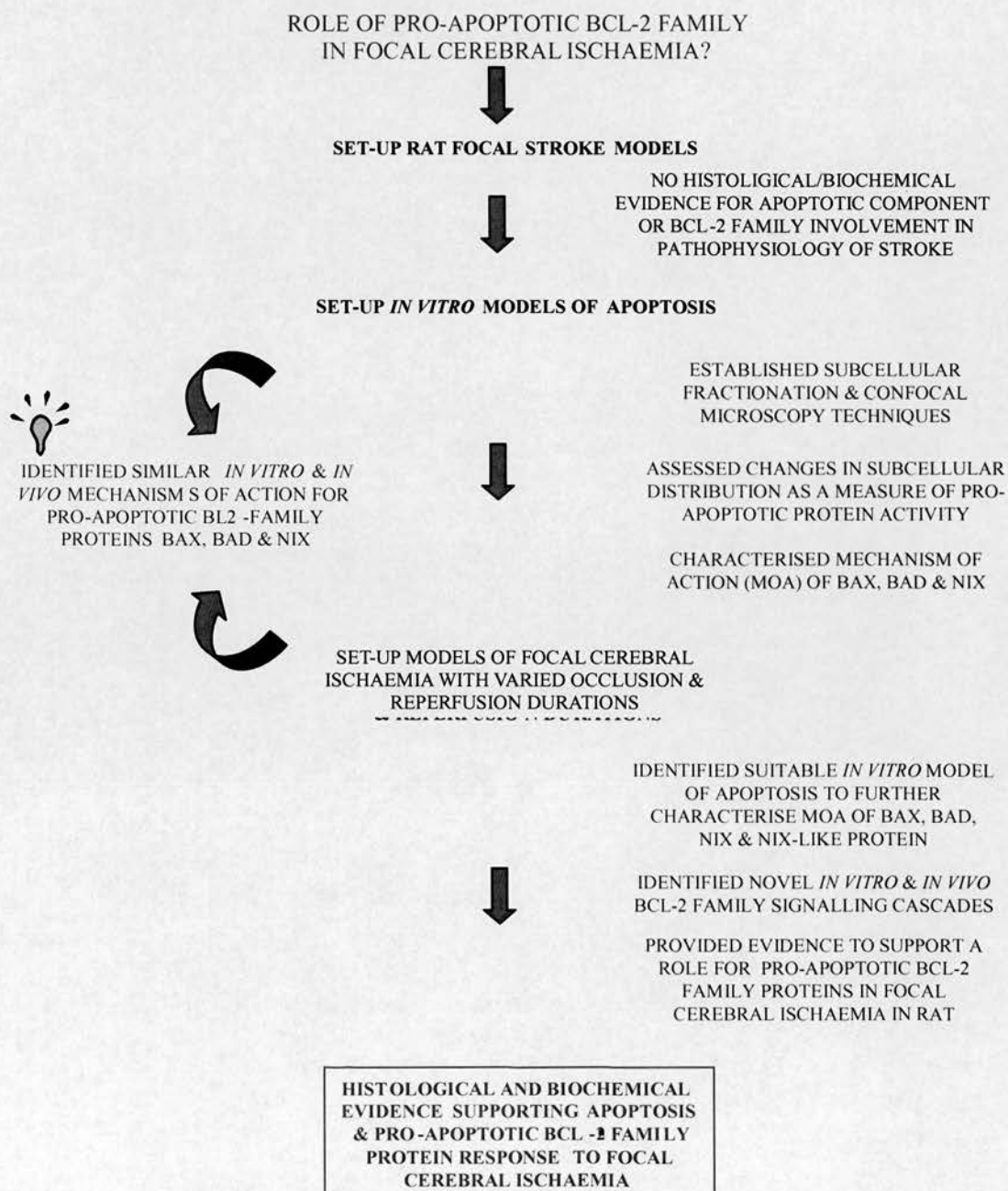


Figure 8.1: Schematic representation of thesis.

MAJOR FINDINGS

RAT STROKE MODELS

Studies described in Chapter 2 were designed to investigate the hypothesis that pro-apoptotic Bcl-2 family proteins Bax, Bad and Nix might be upregulated during focal cerebral ischaemia. Permanent and transient (with reperfusion) stroke model were set-up in the rat using the monofilament middle cerebral artery (MCA) occlusion technique (Koizumi *et al.* 1989). The lesion produced by these two models was similar encompassing the entire MCA territory, and was comparable in volume and distribution of damage to other studies (Tamura *et al.* 1981, Soltanian-Zadeh *et al.* 2003). An apoptotic response was not observed 24 h after onset of ischaemia, as assessed by changes in total protein expression of Bax, Bad and Nix and of cleaved caspase-3 as well as morphological assessment of damage. Other studies have however reported DNA fragmentation using a similar stroke model (Hayashi *et al.* 1999, Davoli *et al.* 2002), although the relevance of such findings has been argued. For example, when caspases are inhibited, cells can still die in response to pro-apoptotic signals without acquiring full apoptotic morphology (Xiang *et al.* 1996, Hirsch *et al.* 1997, Leist *et al.* 1997). In such a case, cells succumb to a bioenergetic catastrophe resulting from mitochondrial dysfunction and/or to caspase-independent pathways, including AIF-mediated large-scale DNA fragmentation (Susin *et al.* 1999). Morphological findings were indicative of a predominantly passive type of cell death, where most neurones appeared, 'ghost-like' indicative of end stages of infarction. Subsequent *in vitro* studies (see below) confirmed analysis of changes in subcellular distribution (e.g. mitochondrial accumulation) as a more sensitive method of assessing pro-apoptotic Bcl-2 family protein activity. However, since no cleaved caspase-3 could be detected in either the core striatal lesion or overlying cortex, it was considered unlikely that apoptosis would play a significant role in cell death in the rat model of transient ischaemic stroke investigated in this initial study. These findings are in keeping with the proposal that apoptotic mechanisms might become 'unveiled' during less severe ischaemic conditions (Choi *et al.* 1996, Chopp *et al.* 1996). Based on initial *in vivo* stroke models data, *in vitro* studies were designed to optimize detection of pro-apoptotic Bax, Bad and Nix in an *in vitro* model of apoptosis using a simplified cell system, with the aim of subsequently using optimized fractionation techniques and less severe focal ischaemia models to investigate the role of apoptosis in ischaemic pathology.

IN VITRO REGULATION OF BAX, BAD, & NIX

In addition to being regulated by protein-specific post-translational modifications, such as cleavage, and phosphorylation, available data suggests mitochondrial accumulation of 'multidomain' and 'BH3 domain-only' members to be a critical event in transducing apoptotic cell death signals and promoting mitochondrial dysfunction. Therefore, prior to characterisation of the activation pathways for pro-apoptotic Bax, Bad and Nix, a reproducible protocol for obtaining enriched mitochondrial and cytosolic fractions was set-up using subcellular fractionation techniques. Staurosporine-treated human SH-SY5Y neuroblastoma cells were chosen to further characterise Bax activation. Following characterisation of *in vitro* Bax activation, studies were performed to investigate whether BH3-only members Bad and Nix could also be activated in the same model of apoptosis. Nix activation was also investigated in a model of hypoxia-induced apoptosis in CHO-K1 cells, where induction of BNip3 and its homologue Nix mRNA, and BNip3 protein expression had been previously reported (Bruick 2000). The regulatory mechanism(s) of each subgroup of pro-apoptotic Bcl-2 family proteins were compared and used to provide a basis for assessing *in vivo* pro-apoptotic changes in the subsequent study.

These studies confirmed that while pro-apoptotic Bax and Nix total protein expression levels changed in response to an apoptotic stimulus (staurosporine and/or hypoxia), protein expression was a poor determinant of pro-apoptotic Bad activity. Translocation of Bax and Bad, from the cytosol to mitochondria, was however observed following an apoptotic stimulus. In the staurosporine model of apoptosis, Bax translocation was associated with a conformational change at the mitochondria. Bax attachment to the outer mitochondrial membrane (OMM) changed from 'attached' to 'inserted', as determined using alkali extraction methods. Moreover, Bax translocation occurred concomitantly with loss of mitochondrial membrane potential and cytochrome c release. These findings are in keeping with other reported data (Antonsson *et al.* 2001) and support a model of Bax-mediated cytochrome c release through pore formation and/or forming a component of an existing pore, such as the permeability transition pore (PTP).

Using the same staurosporine model of apoptosis, Bad activation was investigated. In this model, dephosphorylation of Bad (serine residue 136), translocation to the mitochondria and attachment (but not insertion) of dephosphorylated Bad into the OMM was observed. These findings are in keeping with the proposed mechanism that BH3-only proteins devoid of a transmembrane (TM) domain require other Bcl-2 family proteins to induce cytochrome c release and cell death (Bouillet *et al.* 2002, Letai *et al.* 2002, Puthalakath & Strasser 2002). Since serine 136 of Bad is a substrate for Akt phosphorylation (Datta 1997, del Peso *et al.* 1997, Franke *et al.* 1997), Akt and P⁴⁷³ Akt signalling mechanisms were investigated as an additional site of regulation for Bad. P⁴⁷³ Akt was identified for the first time as both a cytosolic and mitochondrial-associated protein. Apoptosis was associated with

loss of total and P⁴⁷³ Akt immunoreactivity in both the cytosolic and mitochondrial fractions examined. This might reflect a novel survival signalling mechanism, at least in the human neuronal-like cells used in this study. P⁴⁷³ Akt has been reported to phosphorylate Bad on serine residue 136 (Datta *et al.* 1997), which in addition to phosphorylation of additional serine residues by other regulators renders this protein inactive through binding to 14-3-3 scaffolding proteins (Zha 1996, Hsu 1997). Apoptosis in the present study was associated with dephosphorylation of Bad (total Bad expression remained relatively unchanged) and apparent re-distribution of Bad. Akt might have several independent roles in cell survival, one of which being regulation of Bad phosphorylation in the cytosol, and another being regulation of mitochondrial integrity. One proposed mechanism of action for mitochondrial-associated phospho-Akt might involve regulation of oxidative phosphorylation, and in turn, cell integrity. In an ATP dependent process, hexokinase (HXK) mediates the first step in glycolysis, phosphorylating glucose to produce glucose-6-phosphate. In transformed cells, up to 70% of the HXK II is bound to the mitochondria through an interaction with VDAC. At this location, HXK II gains preferential access to mitochondria-produced ATP (Forte 1987, Nakashima 1986, Arora 1988). Over-expression of Akt was observed to increase the levels of mitochondrial-bound HXK (Vander Heiden *et al.* 2001). In contrast, apoptosis-induced detachment of HXK (Gottlob 2001) was associated with concomitant Bax translocation towards the mitochondria (Pastorino *et al.* 2002). In this scenario, mitochondria-associated phospho-Akt reported in Chapters 4, -6 and -7 might have indirectly inhibited mitochondrial accumulation of Bax, and ultimately cell death.

In contrast to other pro-apoptotic Bcl-2 family members such as Bax and Bad, a marked induction of BNip3 and Nix mRNA expression was recently reported in response to hypoxia in several cell lines, including Chinese Hamster Ovary (CHO-K1) cells (Bruick 2000, Regula *et al.* 2002). Unlike other family members, BNip3 and Nix contain a hypoxia responsive element (HRE) in their promoter sequence. During hypoxia, the transcription factor, HIF-1 α , is stabilised which induces the transcriptional activation of HRE-containing genes, such as BNip3 and Nix (Bruick, 2000). Therefore, it was suggested that these two proteins might have a specific role to play in hypoxia-induced cell death, and possibly during focal cerebral ischaemia. A definitive experiment by Bruick (2000) demonstrated de novo synthesis of a 60 kDa dimer of the BNip3 protein in hypoxic CHO-K1 cells, however Nix protein expression was not examined (Bruick 2000). Studies were therefore designed to characterise pro-apoptotic Nix protein activation in hypoxic CHO-K1 cells and to compare the activation pathway with that reported for BNip3. Initial attempts to reproduce Bruick's experiment failed. Specific BNip3 protein expression could not be detected using antibodies available at the time of study; anti-serum (*gift from Richard Bruick*) or anti-BNip3 (Santa Cruz) failed due to non-specific binding and excessive background. In contrast, the Nix antibody identified two immunoreactive bands which, based on sequence homology between BNip3 and Nix, were proposed to correspond to Nix (48 kDa) and to a novel 30 kDa Nix-like protein or monomer of BNip3. Antibody specificity to both immunoreactive bands was confirmed using K562 cells provided as a positive control with the

antibody. Initial studies reported BNip3 (Chen *et al.* 1997) and Nix (Chen *et al.* 1999) as primarily mitochondrial-associated proteins. These findings were of particular interest, since all other BH3-only members to date have been reported to display either a cytosolic (or cytoskeletal) distribution pattern in control cells. Therefore, it was anticipated that BNip3 and Nix might directly or indirectly regulate mitochondrial integrity, specifically during conditions of hypoxia, such as during focal cerebral ischaemia. However, published studies used over-expression techniques and therefore might not accurately reflect the subcellular localization of the endogenous proteins. Studies were therefore designed to characterise hypoxia-mediated regulation of Nix protein expression, with the aim of subsequently investigating whether Nix (or the 30 kDa Nix-like protein) could be activated by stimuli other than hypoxia.

Data presented in Chapter 5 demonstrated that in untreated CHO-K1 cells, Nix and the 30 kDa immunoreactive Nix-like protein were observed as primarily cytosolic and mitochondrial-associated proteins, respectively. This agrees with recent findings (Vande Velde *et al.* 2000) that suggested BNip3 and its homologue Nix were distinct regulators of hypoxia-mediated cell death, and as such, should be considered as individual Bcl-2 members. Studies described in Chapter 5 identified that apoptosis was associated with upregulation/de novo synthesis of both Nix and Nix-like proteins. A similar subcellular distribution and pro-apoptotic response was observed in staurosporine-treated human SH-SY5Y neuroblastoma and dexamethasone-treated mouse A1.1 monocyte cells. These are the first studies to demonstrate Nix and Nix-like protein activation in response to apoptotic stimuli, other than hypoxia. These findings suggest that Nix, BNip3 and/or the Nix-like protein might regulate more types of apoptotic cell death than originally considered. The precise upstream regulators of these proteins remain unclear, since the exact mechanism(s) of staurosporine and dexamethasone-mediated apoptosis remain incompletely defined. Recent findings support a role for ROS-mediated activation of HIF-1 α in lung epithelia (Hadaad *et al.* 2001), and might therefore represent a novel signalling mechanism for Nix and /or the Nix-like protein following non-hypoxic stimuli. Also, p53-mediated upregulation of Bax (Schuler *et al.* 2003) and BNip3 (Tsunoda *et al.* 1999) has been reported in some but not all systems (Guo *et al.* 2001), further supporting these two signalling mechanisms as possible mediators in the observed Nix and 30 kDa Nix-like protein responses to staurosporine and dexamethasone treatment in the present study. Interestingly, translocation of Nix towards the mitochondria was not observed in either *in vitro* model of apoptosis investigated. This might reflect low endogenous levels of Nix protein in SH-SY5Y and A1.1 cells. Mitochondrial levels of endogenous Nix protein might have been below the limits of detection in these studies. The reverse might apply for the primarily mitochondrial Nix-like protein. It is possible that insufficient apoptotic stimuli in these models might have prevented Nix translocation, although this seems unlikely as other Bcl-2 family proteins, Bax, Bad and the 30 kDa Nix-like protein were post-translationally modified under the same experimental conditions.

Chapters 3, -4 and -5 provided evidence supporting a model where several BH3-only as well as multidomain pro-apoptotic Bcl-2 family proteins are activated in a given *in vitro* model of apoptosis. Also, at least following staurosporine treatment, apoptosis is associated with translocation/mitochondrial accumulation of Bax, Bad and the 30 kDa Nix-like protein, and accompanied by inner mitochondrial membrane depolarisation and concomitant cytochrome c release. Data from these studies argues against a direct role for Nix in promoting the observed changes in cytochrome c distribution, since mitochondrial accumulation of this protein was not observed. While Bad attachment to, and Bax insertion into, the outer mitochondrial membrane (OMM) was demonstrated, attempts to characterise the interaction of the Nix-like protein at the mitochondria failed and this warrants further investigation. Elucidation of this interaction might enable better characterisation of the precise control pro-apoptotic Bcl-2 family sub-groups have over mitochondrial integrity. Insertion of Bax and the 30 kDa Nix-like protein into the OMM, as has been previously reported for BNip3 (Ray *et al.* 2000), would support the hypothesis that multidomain and BH3 / TM domain-containing members might promote mitochondrial dysfunction and cytochrome release through direct insertion into the mitochondrial membrane. In contrast, BH3-only proteins devoid of a TM domain required for anchoring to the outer membrane (such as Bad) can attach to but not insert into the OMM. In this scenario, Bad-like members might bind to anti-apoptotic proteins and displace *multidomain* Bax-like molecules; thus indirectly promoting cytochrome c release (Letai *et al.* 2002). Data presented in this thesis supports such a model, since Bad appeared attached, but not inserted into the OMM of apoptotic SH-SY5Y cells (*Chapter 4*). A third group of proteins containing a BH3 and TM domain, such as Bim, BNip3 and Nix, have been reported to insert into the OMM. Bim has also been recently reported to directly bind and activate Bax (Yamaguchi *et al.* 2002). Ray *et al.* (2000) reported that BNip3 inserts into the OMM following transient over-expression in HeLa cells, these findings support such a mechanism of regulation for the BH3-only proteins which contain a TM domain. However, several recent reviews of Bcl-2 family have failed to comment on the regulatory mechanisms of either Nix or BNip3 regulation (Bouillet *et al.* 2002, Letai *et al.* 2002, Puthalakath *et al.* 2002). Elucidation of whether Nix, BNip3 and/or the Nix-like protein bind Bax-like, Bcl-2-like or other proteins at the OMM would facilitate characterisation of these *BH3-only* proteins and their specific role(s) in Bcl-2 family-mediated cell death mechanisms. Nevertheless, data from this thesis together with other studies (Hsu & Youle *et al.* 1997, Letai *et al.* 2002) support a model where the *BH3-only* proteins and *multidomain* proteins have distinct, but interdependent functions essential for initiation and transduction of the apoptotic signal. However, whether these two main subgroups of pro-apoptotic proteins are part of the same pathway or act in parallel, both impinging on the Bcl-2-like pro-survival proteins is presently not clear.

STAUROSPORINE-MEDIATED MITOCHONDRIAL DYSFUNCTION & CYTOCHROME C RELEASE

Studies were designed to characterise the mechanism of cytochrome c release and the putative role of the mitochondria in staurosporine-treated human SH-SY5Y neuroblastoma cells. Staurosporine-mediated apoptotic cell death was associated with Bax translocation and insertion into the outer mitochondrial membrane with simultaneous inner mitochondrial membrane depolarization and cytochrome c release. Interestingly, using confocal microscopy, residual mitochondrial staining of cytochrome c as well as residual mitochondrial membrane polarity was observed after 5 h staurosporine treatment; a time point where most cells appeared shrunken and at the end stages of cell death. Cytochrome c release was quantified using an ELISA assay and confirmed findings from confocal microscopy studies, supporting a more gradual release of cytochrome c during staurosporine-mediated apoptosis. This sub-maximal release of cytochrome c and residual membrane polarity supports an active role of the mitochondria in apoptotic cell death, where based on findings in these studies, it might be anticipated that residual ATP generation enabled execution of the mitochondrial (intrinsic) apoptotic cell death pathway. Since residual ATP levels were reported during both permanent and transient focal cerebral ischaemia (Hata *et al.* 2000b, Sims *et al.* 2003), these findings support a role for activation of this intrinsic cell death pathway in ischaemic cell death (see below). Considering that apoptosis is an ATP consuming process (Leist *et al.* 1997), it is plausible that the fraction of cytochrome c released early in the process might participate in apoptosome formation, whereas the cytochrome c portion that remains mitochondrially-associated would temporally warrant sustained ATP production. The initially released pool of cytochrome c might be the soluble fraction from the intermembrane space whereas the second pool might comprise the fraction more tightly associated with the inner mitochondrial membrane (Ott *et al.* 2002, Scorrano *et al.* 2002). Over time, progressive damage to mitochondria would however become irreversible, ensuring cell death. The 'point-of-no-return' therefore might not be an abrupt phenomenon but rather a process accumulating in the decisive disruption of the mitochondrial membrane potential. Graded cytochrome c release with only partial IMM depolarisation was observed in the present *in vitro* study using staurosporine-treated SH-SY5Y neuroblastoma cells. Therefore, it might be predicted that residual ATP production enabled activation of downstream caspases via formation of the apoptosome in these studies. Supporting this proposal, increased cleaved caspase-9 immunoreactivity was detected in apoptotic compared to control SH-SY5Y cells.

Despite these findings, a central role for mitochondrial disruption in apoptotic cell death is difficult to reconcile with the lack of any evidence for the involvement of cytochrome c in cell death in *C. elegans*. Furthermore, although *Drosophila* contain an essential APAF-1 ortholog with WD40 repeats (DARK), apoptosis in the fly does not appear to require cytochrome c (Dorstyn *et al.* 2002,

Zimmermann *et al.* 2002). Initial knockout studies reported that mice lacking either APAF-1 (Cecconi *et al.* 1998, Yoshida *et al.* 1998) or caspase-9 (Kuida *et al.* 1998, Hakem *et al.* 1998) often died before birth and had enlarged brains, and apoptosis of several cell types was impaired *in vitro* (Kuida *et al.* 1998, Hakem *et al.* 1998, Yoshida *et al.* 1998). However, post-mitotic neurones that lack APAF-1 were reported to still die by apoptosis (Honarpour *et al.* 2001), and some APAF-1^{-/-} mice become healthy adults (Honarpour *et al.* 2000). Indeed, caspase-dependent apoptosis can occur without cytochrome c release (Holinger *et al.* 1999, Li *et al.* 1999), whereas certain cells remain viable for days after disruption of the mitochondrial outer membrane (von Ahsen *et al.* 2000). Therefore, measurement of apoptotic cell death by cytochrome c release might not truly reflect apoptotic activity.

IN VIVO MODELS OF FOCAL CEREBRAL ISCHAEMIA

In vitro and *in vivo* studies have previously reported changes in mRNA expression of various pro- and anti-apoptotic mediators with comparable ease. However, it remains essential to study protein changes as a measure of cellular activity. The functional relevance of gene responses to focal cerebral ischaemia has been debated, since changes in mRNA expression do not necessarily reflect changes in protein expression: this event requires protein synthesis, which was reported to be severely suppressed in at least some models of focal cerebral ischaemia (Mengesdorf *et al.* 2002). However, it should be noted that increased protein expression does not necessitate concomitant functional changes in a given protein. No changes in Bax, Bad and Nix or cleaved caspase-3 protein expression were detected in early stroke studies (Chapter 2). Subsequent *in vitro* studies confirmed subcellular fractionation as a more accurate measure of protein activity (Chapters 3, -4 and -5). Therefore Chapters 6 and -7 investigated the effects of varied occlusion and reperfusion durations on Bax, Bad, Nix and cleaved caspase-3 protein expression levels and subcellular distribution. These studies focused on early time points and a shorter duration of ischaemia for the study of apoptotic cell death in focal cerebral ischaemia. Based on available data at the time of study (Du *et al.* 1996, Gillardon *et al.* 1999, Kitagawa *et al.* 1999), it was anticipated that these less severe ischaemia models might 'unveil' any apoptotic response which had occurred (Choi *et al.* 1996).

Importantly, regulatory mechanisms observed for each protein *in vitro* were also demonstrated *in vivo*. For example, Bax, Nix and the 30 kDa Nix-like protein, but not Bad, exhibited ischaemia-mediated changes in total protein expression and mitochondrial accumulation. Interestingly, in untreated SH-SY5Y cells Nix and the Nix-like protein were only observed in the cytosol and mitochondria, respectively. This is in contrast to *in vivo* studies, where Nix still appeared primarily cytosolic, however the 30 kDa Nix-like protein was detected in almost equal amounts in the cytosol and mitochondria, of sham rat cortex and striatum. This might reflect greater endogenous protein

expression levels in rat brain compared to the cell lines investigated. Alternatively, SY-SY5Y cells are transformed and therefore might not accurately reflect endogenous protein expression patterns. Prolonged ischaemic duration and apoptotic cell death (as measure by cleaved caspase-3 and appearance of apoptotic bodies) was associated translocation of Bax, Nix and the Nix-like protein. As with *in vitro* studies, increased ischaemic cell death was observed with concomitant and complete dephosphorylation of Bad on serine 136 and reductions in the levels of cytosolic Bad. In contrast to *in vitro* studies, concomitant accumulation of dephosphorylated Bad could not be detected in the ischaemic rat brain. However simultaneous changes in Bad and Akt suggest that Bad translocation, although undetectable, likely occurred in this experimental paradigm. Cumulatively, these findings support the use of *in vitro* models of apoptosis, at least the staurosporine model used in the present study, to further characterise the molecular circuitry of this family of regulatory proteins.

INCREASED BAX, NIX AND NIX-LIKE RESPONSE WITH INCREASED DURATION OF ISCHAEMIA AND REPERFUSION.

In permanent and transient (1 h) models of focal ischaemia, a progressive increase in pro-apoptotic Bax, Nix and Nix-like protein expression was observed between 6 h and 24 h after ischaemic onset. However, a delayed response was observed for each protein in the transient compared to permanent ischaemia models suggesting that reperfusion delayed the progression of cell death, but could not alter the ultimate cell fate. Interestingly, while Nix and Bax translocation towards the mitochondria were observed in the core striatal lesion after 24 h permanent ischaemia, translocation of Nix could only be detected in the less severely damaged cortex at this time point. These findings suggest that, at least under conditions of focal cerebral ischaemia used in the present study, Nix translocation might precede Bax translocation. In this scenario, Nix would bind to the mitochondria and facilitate some event in mitochondrial accumulation and/or insertion of Bax. Future investigation and characterization of Nix:Bax interactions *in vivo* and *in vitro* will no doubt facilitate elucidation of the mechanisms of action of these pro-apoptotic Bcl-2 family proteins and their specific role in different models of apoptotic cell death. Interestingly, yeast two-hybrid analysis in human 293T cells recently identified an additional site of regulation for pro-apoptotic Bad (Won *et al.* 2003). In this study, 14-3-3 epsilon protein was identified as a substrate for caspase-3. Caspase-3-mediated cleavage of 14-3-3 epsilon at asparagine 238 was reported to facilitate Bad association with Bcl-X_L more effectively, thus promoting cell death. Whether such a feed-forward amplification mechanism might exist *in vivo*, remains to be investigated. Interestingly, 14-3-3 epsilon was recently reported to directly bind Bax, where caspase-dependent and independent cleavage of 14-3-3 and release of Bax was observed. However, the functional implications of such findings await further investigation (Nomura *et al.* 2003).

While caspase inhibition is neuroprotective in models of both transient and permanent focal ischaemia (Hara *et al.* 1997, Weissner *et al.* 2000, Chen *et al.* 2002), the protection exceeded that which could be expected if only cells exhibiting the morphological features of apoptosis were salvaged. This finding is consistent with the possibility that apoptotic processes may be activated in some cells without complete progression to the classical morphological outcome. Increasing findings suggest that the classical definitions of apoptosis and necrosis might not apply when describing focal ischaemic damage. In addition, while apoptosis and necrosis were originally identified by virtue of their distinct morphologies (Kerr *et al.* 1972), these two cell death pathways were recently proposed to form part of a continuum involving similar biochemical mediators (Roy & Sapolsky 1999). In support of this proposal, analysis of DNA fragments from focal ischaemic tissue is reported to display 'staggered', rather than the 'blunt' ends, characteristic of classical developmental apoptosis. These findings support a distinct form of cell death during focal cerebral ischaemia, from what is classically defined as either apoptosis or necrosis (Kuschinski & Gillardon 2000). Although less well-understood, a third potential cell death pathway, involving autophagy, has also been implicated in the pathology of global ischaemia (Lemasters *et al.* 2002) and might also be involved in focal ischaemia. Data presented in this thesis supports such an admixed morphology, and/or potential merging of biochemical cell death pathways in focal cerebral ischaemia. After 24 h permanent and transient ischaemia, the core infarct was surrounded by a mixture of end-stage necrotic and apoptotic cells. Biochemical and histological findings suggest that while preferentially entering the energy-consuming intrinsic mitochondrial apoptotic cell death cascade, with prolonged ischaemia the final execution of cell death might have involved a more necrotic and passive type of cell destruction.

Biochemical (pro-apoptotic Bax, Bad, Nix and Nix-like protein expression and mitochondrial accumulation, increased cleaved caspase-3 immunoreactivity) and morphological (apoptotic bodies, cell shrinkage) changes indicative of apoptotic cell death were observed in both permanent and transient models of focal ischaemia. Interestingly, in these studies the greatest pro-apoptotic response was observed in the most severely damaged tissue and in following the most severe ischaemic paradigm; 24 h permanent MCA occlusion. These findings provided strong evidence to support a role for apoptosis in ischaemic cell death, at least under these experimental conditions. Data obtained in this thesis strongly support an active role for apoptotic mechanisms in the pathology of focal cerebral ischaemia in the rat. However, upstream activators, or ligands for these pro-apoptotic Bcl-2 family proteins remain elusive. Overall these findings provide a strong basis for arguing the relevance for the use of less severe models of focal cerebral ischaemia in attempts to delineate the pathophysiology of stroke. However, an overhanging question still remains for many ischaemic mediators: is the observed response part of the injurious outcome, or part of an adaptive repair response to the initial ischaemic insult (which may be protective during a second ischaemic event; preconditioning phenomenon)?

Future studies might involve characterisation of the mechanism of action of pro-apoptotic Bax, Bad, Nix and the Nix-like protein at the outer mitochondrial membrane in the ischaemic brain. However, should experimental verification sustain verbal speculation, such mitochondrial events would support an active role for the mitochondria in apoptotic cell death mechanisms during focal cerebral ischaemia. A proposed mechanism whereby some cytochrome c remains bound to the mitochondrial to maintain residual ATP production, might explain why increased cleaved caspase-9 immunoreactivity (Wang *et al.* 2002, Pfister *et al.* 2003) and the protective effects of inhibiting this protein (Mouw *et al.* 2002) have been reported during focal cerebral ischaemia and in the *in vitro* studies described in this thesis (*Chapter 3*). Furthermore, early cytochrome c release has been reported following permanent (Pfister *et al.* 2003) and transient (Wang *et al.* 2002) ischaemia. Together, these findings further support a role for mitochondria-mediated cell death mechanisms during ischaemia.

INTRINSIC AKT / BAD SURVIVAL RESPONSE – EARLY DURING ISCHAEMIA & WITH PROLONGED REPERFUSION

In contrast to the progressively increased pro-apoptotic response of Bax, Nix and the Nix-like protein observed with increased ischaemic and reperfusion duration, an intrinsic survival-like response to ischaemia and reperfusion was reflected in changes in Bad activity. An early inhibition of Bad was observed after 6 h permanent ischaemia and with increased reperfusion duration. This was demonstrated by increased phosphorylation of serine residue 136 on Bad and increased cytosolic levels of total Bad. Since *in vitro* studies (*see Chapter 4*) demonstrated that Akt signalling correlates with Bad activity, a novel regulatory mechanism was proposed for Akt signalling at the mitochondria of healthy cells.

Studies presented in Chapters 6 and -7, investigated Akt response(s) to increased ischaemic and reperfusion durations in attempts to further characterise the observed Bad response. As observed using an *in vitro* model of apoptosis (*Chapter 4*), P⁴⁷³ Akt was detected in both the cytosol and mitochondria-enriched fractions of sham animals. P⁴⁷³ Akt translocation towards the mitochondria was observed early during permanent ischaemia and with prolonged reperfusion, indicative of a novel Akt signalling mechanism. This was supported by good neuronal preservation in the cortex, and less so, striatum even after 24 h transient (1 h) ischaemia. As discussed in Chapter 8, these findings contradict literature which suggests 24 h transient ischaemia is a sufficient stimulus to produce a mature ischaemic lesion encompassing the entire MCA territory (Gillardon *et al.* 1996). However, Gillardon *et al.* also reported a cortical lesion following 7 h transient (1 h) ischaemia in rats, suggesting that slight discrepancies might exist in Gillardon's protocol and the protocol described in Chapters 6 and -7. Biochemical and histological data from permanent and transient models of ischaemia in this thesis appeared consistent. In each study, an increased pro-apoptotic response correlated with increased

ischaemic duration. However, 24 h transient ischaemia was associated with a comparatively delayed pro-apoptotic Bax, Nix and Nix-like response and cleaved caspase-3 immunoreactivity compared to 24 h permanent ischaemia. Also, after 24 h transient ischaemia, comparatively improved cell preservation was observed in the ischaemic hemisphere, with an Akt-mediated inhibition of the pro-apoptotic Bad response. This is the first study to report such a signalling mechanism for Akt both *in vitro* and *in vivo*, and importantly, the first study to correlate Akt survival signalling with inhibition of pro-apoptotic Bad and delayed ischaemic damage during ischaemia/reperfusion in the rat.

In contrast, loss of all detectable Akt staining was reported in both the cortex and striatum after 24 h permanent ischaemia. Also, loss of mitochondrial P⁴⁷³ Akt was associated with dephosphorylation and reduced cytosolic levels of Bad (indicative of Bad re-distribution), Bax translocation, increased cleaved caspase-3 immunoreactivity and increased detection of apoptotic bodies. These findings are in agreement with *in vitro* data (see Chapter 4), and further support a novel regulatory mechanism for P⁴⁷³ Akt at the mitochondria. *In vitro* studies reported that Akt can directly phosphorylate and inactivate both Bad (Datta *et al.* 1997, Datta *et al.* 2002) and caspase-9 (Cardone *et al.* 1998). More recently, Akt was proposed to regulate Bax translocation (Tsuruta *et al.* 2002, Yamaguchi *et al.* 2002). However, as no Akt phosphorylation sites have been identified in Bax, it therefore seems unlikely that Akt directly regulates Bax activity. Interestingly, Bax and Bad translocation was only detected after 24 h permanent ischaemia (Chapter 6); a time point where loss of all detectable Akt and concomitant increased caspase-3 immunoreactivity were reported. Therefore, it might be proposed that mitochondrial-localised Akt, observed after 6 h permanent ischaemia (Chapter 6) and with prolonged reperfusion (Chapter 7) might directly regulate mitochondrial integrity. Several possible mechanisms of action for mitochondrial-bound Akt exist: (i) regulation of oxidative phosphorylation (Gottlob *et al.* 2001), (ii) Akt might bind some, as yet, undefined pro-apoptotic Bcl-2 family protein receptor, which in turn blocks the targeting signal for Bax-like molecules, (iii) Akt might provide steric hindrance at this receptor where loss of Akt at the mitochondria might permit mitochondrial accumulation of Bax and/or other pro-apoptotic Bcl-2 family proteins. *In vitro* studies have reported caspase-9 release from the mitochondria following an apoptotic stimulus. Caspase-9 inhibition was reported to reduce infarct size (Mouw *et al.* 2002) and therefore (iv) mitochondrial P⁴⁷³ Akt might phosphorylate and inhibit mitochondrial release of caspase-9. In this scenario, Akt-mediated phosphorylation of caspase-9 might also render this apoptotic mediator inactive by sequestering it in the mitochondria, thus preventing its release into the cytosol and cleavage of downstream effector caspases, such as caspase-3.

Alternatively, Akt might indirectly regulate glycolysis by promoting hexokinase association with VDAC at the mitochondria. It has been proposed that hexokinase might induce VDAC to adopt a conformational state in which Bax is unable to bind (Ouyang *et al.* 1999, Plas *et al.* 2001, Pastorino *et al.* 2002). In this scenario, apoptosis or prolonged focal cerebral ischaemia (Chapter 6) might result in

loss of mitochondrial-bound P473 Akt, with concomitant loss of hexokinase regulation of Bax, which in turn, might allow Bax translocation towards the mitochondria. Bax might in turn bind VDAC to promote cytochrome c release. The implications of such an event remain incompletely understood, although interesting because a Bax:VDAC interaction has been reported sufficient to allow passage of cytochrome c *in vitro* (Shimizu *et al.* 2000, Tsujimoto *et al.* 2002).

Bax might bind to other components of the PTP to facilitate cytochrome c release and apoptotic cell death. *In vitro* studies described in Chapter 3 support the hypothesis that the mechanism of Bax-mediated cytochrome c might be dependent on the concentration of Bax at the mitochondrial membrane (Pastorino *et al.* 2000). In this scenario, under conditions of focal cerebral ischaemia applied in this thesis, the mechanism of cytochrome c release might be dependent on the severity of ischaemic insult, and therefore the concentration of mitochondria-associated Bax in the cortex and striatum in each experimental model. Since *in vitro* studies support a role for Bax-mediated membrane depolarisation, possibly through interaction with one or more components of the PTP, the precise regulatory mechanisms controlling Akt survival activity require further investigation, as does the consequences of observed Bax translocation in ischaemic brain tissue. Investigation of protein:protein interactions of Bax, Akt, VDAC, HXK and Bad, at the mitochondria will no doubt facilitate characterisation of the intrinsic survival mechanisms in permanent and transient focal cerebral ischaemia described in this thesis. Nonetheless, findings from this thesis support a role for Akt as a potential therapeutic target in stroke research. Since, the *in vitro* and *in vivo* mechanism(s) of action of each pro-apoptotic Bcl-2 family protein appeared identical; a schematic diagram of putative mechanism of action of Bax, Bad, Nix and the Nix-like protein has been formulated. Additional published data will be included to support the proposed response of Bax, Bad, Nix, Nix-like (30 kDa) protein as well as Akt and cleaved caspase-3, to permanent and transient focal cerebral ischaemia (Figure 8.2).

(A) SHAM ANIMALS

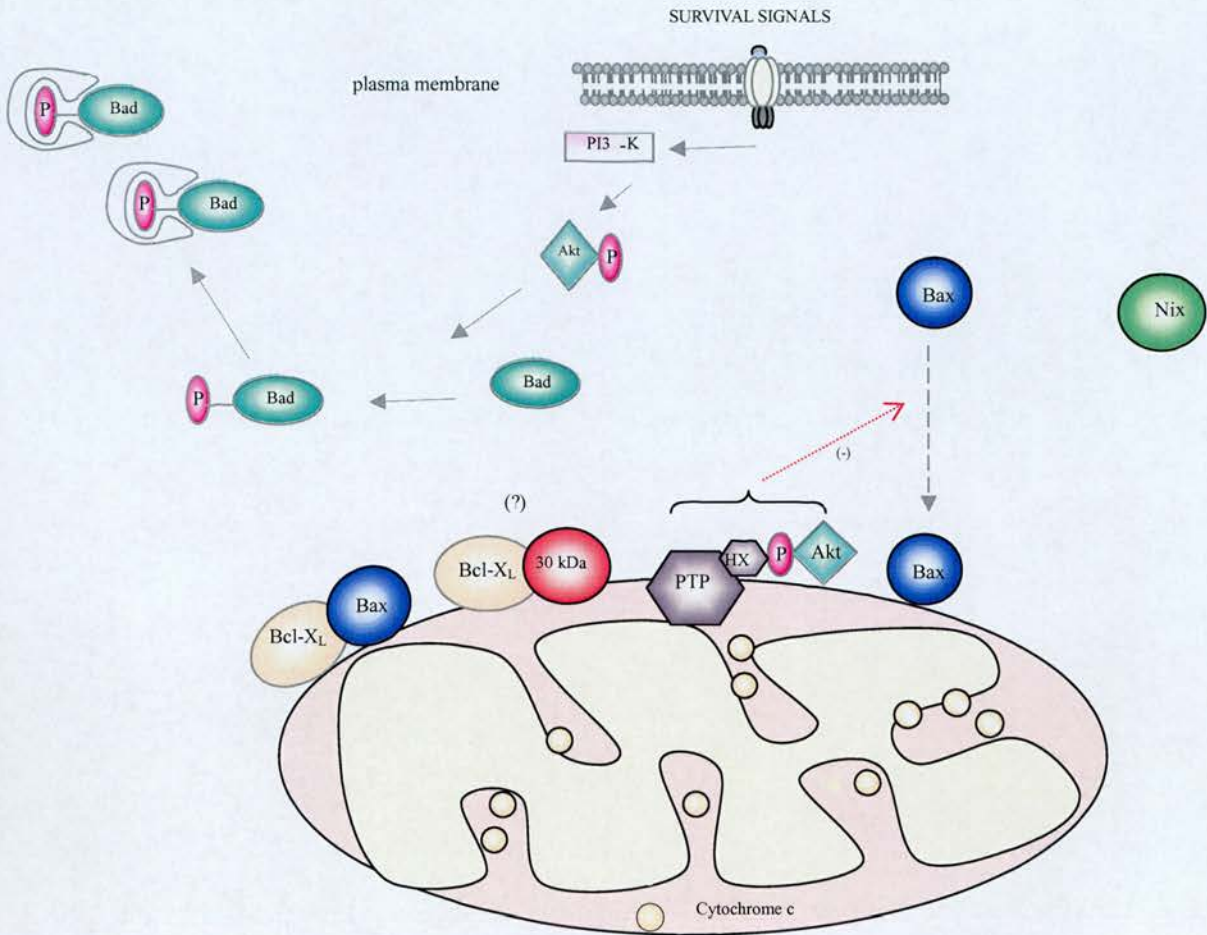


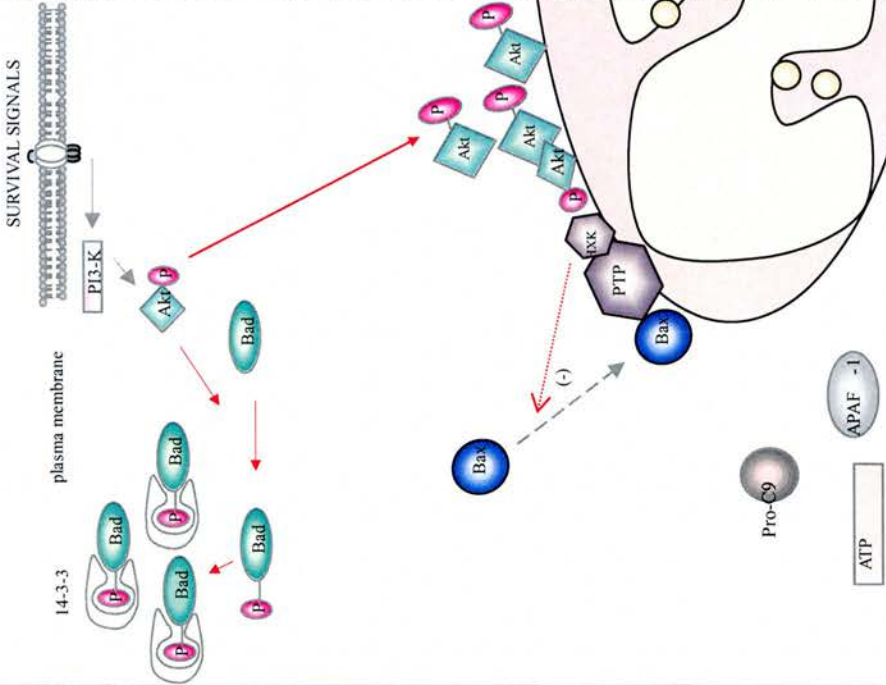
Figure 8.2: Schematic diagram representing observed pro-apoptotic Bcl-2 family response to focal cerebral ischaemia in the rat.

Distribution of endogenous Bax, P136 Bad, Nix, Nix-like (30 kDa) proteins and P473 Akt in sham animals (A). Bax, Nix and Bad are primarily cytosolic proteins. Bax was also detected in the mitochondria and the 30 kDa Nix-like protein appeared both cytosolic and mitochondrial in sham animals. Bad is phosphorylated and bound by 14-3-3 in an inactive state. Mitochondrial-bound Bax is thought to be bound and inactivated by anti-apoptotic Bcl-XL. P⁴⁷³ Akt was observed in both the cytosol and attached to, but not inserted into, the mitochondria. Akt has been reported to regulate hexokinase (HXK) association with VDAC, a component of the permeability transition pore (PTP), which in turn has been reported to regulate Bax subcellular distribution. Akt-mediated survival signalling observed following increased reperfusion duration and early during permanent ischaemia (B). In addition to increased phosphorylation, and thus, inactivation of P¹³⁶ Bad, P⁴⁷³ Akt translocation towards the mitochondria was reported. This might represent a novel regulatory role for Akt at the mitochondria – regulation of mitochondrial integrity and possibly indirect inhibition of Bax. Increased ischaemic duration was associated with loss of all Akt immunoreactivity, with concomitant mitochondrial accumulation of Bax, Bad, Nix and increased mitochondrial expression of the 30 kDa Nix-like protein with concomitant increased levels of cleaved caspase-3 and cell death (C) Progressive pro-apoptotic Bax, Bad, Nix & Nix-like response observed with increase caspase-3 cleavage, lesion expansion and morphological features of apoptotic cell death.

(B)

REPERFUSION / RESIDUAL ENERGY

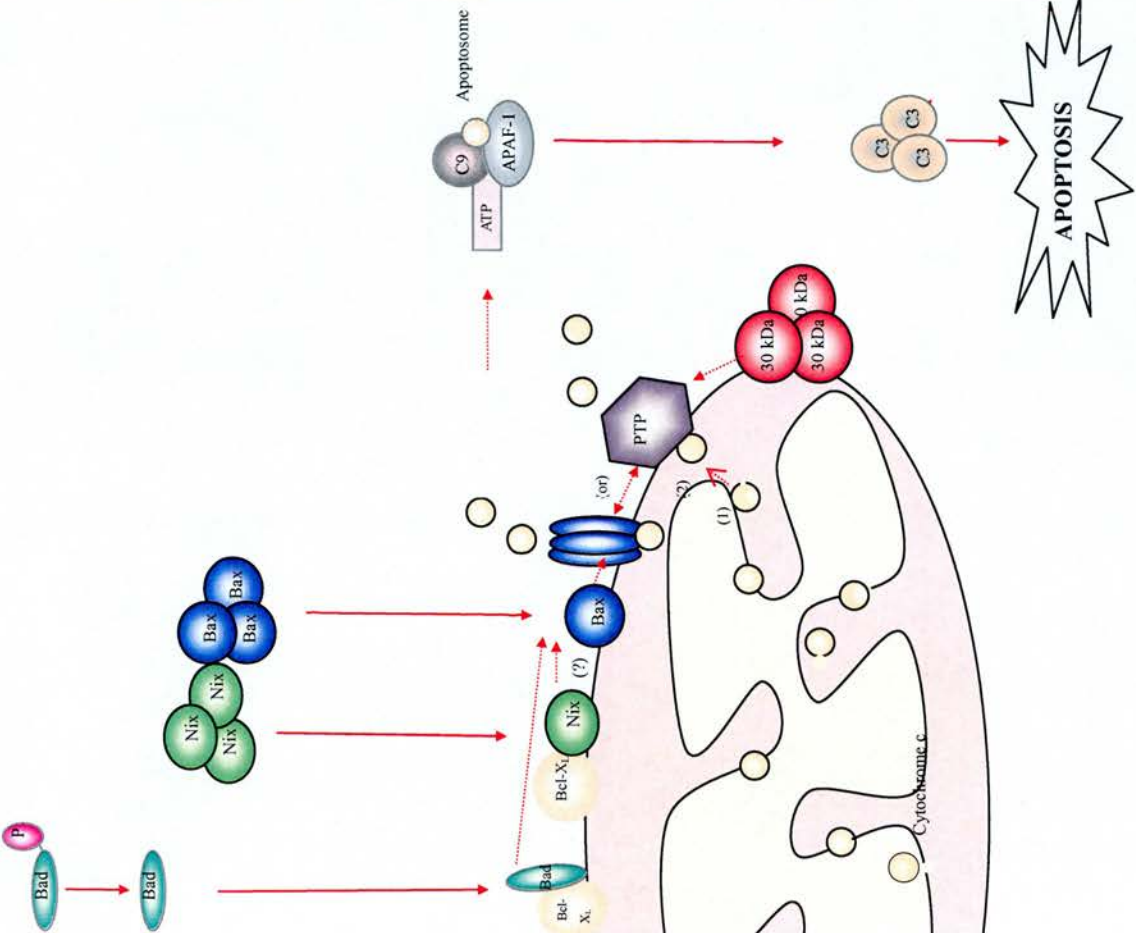
Akt-mediated survival response



(C)

INCREASED ISCHAEMIC DURATION

Pro-apoptotic Bax, Bad, Nix & Nix-like



OPEN QUESTIONS AND OUTLOOK

Many questions arise from this work, which were unfortunately outside the limits of this thesis. It is anticipated that future investigation of these unresolved issues will explain several of the novel findings obtained in this thesis, which at present cannot be fully explained. These include: (a) Does Akt detachment from the mitochondria precede mitochondrial accumulation of Bax in staurosporine-treated SH-SY5Y cells?, (b) Since Nix translocation towards the mitochondria was detected before Bax translocation in the ischaemic cortex, do BH3-only members target/recruit Bax to the mitochondria or do they facilitate Bax insertion into the OMM?, (c) Can Akt over-expression rescue cells and prevent Bax and Bad translocation as well as prevent mitochondrial dysfunction during focal cerebral ischaemia?, (d) What do Nix, BNip3 and/or the Nix-like protein bind to at the outer mitochondrial membrane and does this molecule regulate their activity?, (e) Do Nix homologues (with a TM) act in a similar manner to other TM-containing proteins such as Bim? For example, does Nix require binding to Bax-like or Bcl-2-like proteins to promote cytochrome c release and apoptotic cell death?, (f) What does P⁴⁷³ Akt bind to at the outer mitochondrial membrane and does this molecule regulate Bax targeting to and/or activity at the mitochondria? and finally (g) Which cell type expressed the observed pro-apoptotic changes following permanent and transient ischaemia?

REGULATION OF MULTI-DOMAIN AND BH3-DOMAIN ONLY PROTEINS?

Multiplicity and probable redundancy of pro- as well as anti-apoptotic Bcl-2 family members in mammals has made it difficult to discern their physiologically relevant interactions. Theoretically, specificity could be generated by preferential binding of particular BH3-only proteins to particular anti-apoptotic Bcl-2-like molecules. There is no evidence for or against this theory and it is likely that clarification of this important issue requires careful assessment of the affinities of these protein-protein interactions. Alternatively, specificity of protein interaction could simply be a reflection of their protein expression patterns. All BH3-only proteins investigated to date require the presence of Bax/Bak-like multidomain family members for their ability to execute cell death. In turn, Bax/Bak-like proteins require a cell death signal from BH3-only proteins, which sense and are activated by stress signals (Cheng *et al.* 2001, Zong *et al.* 2001). Comparison of various α -helical peptides from BH3-only Bcl-2 family proteins suggests that this subgroup can be divided into two functional classes: Bid-like proteins (containing a TM domain) were observed to directly activate Bax-like, multi-domain members and Bad-like proteins (without a TM domain) which are proposed to sensitise mitochondria for apoptosis by occupying the binding pocket of anti-apoptotic Bcl-2 or Bcl-XL (Letai *et al.* 2002). Based on such findings it might be anticipated that Nix,

a TM-domain containing BH3-only protein promotes cytochrome c release through direct binding of Bax. However, reports of such a cell death mechanism have not been reported. One caveat to this proposed control over Bcl-2 family proteins is that BNip3 and Nix, BH3-only proteins, have not been investigated with respect to the necessity for a multidomain protein in their cell death pathways. Generation and analysis of mutant mice lacking individual proteins or combinations of these 'killers' will provide an interesting and better understanding of these family members and their specific role in apoptotic regulation. Alternatively 'knock-in' models of mutant mice for various pro-apoptotic Bcl-2 family proteins or the various reported splice variants, would also aid characterisation of this complex family of proteins and their individual and/or synergistic control over apoptotic cell death. Whether Nix, a TM domain-containing BH3-only member, can directly induce cytochrome c when added exogenously to isolated mitochondria, or whether this protein requires binding to multidomain Bax-like proteins to mediate cytochrome c release and apoptotic cell death, requires further investigation.

A key unresolved issue regards which upstream mediators regulate the Bcl-2 family of proteins. The transcription factor p53, and reactive oxygen species, have been proposed to activate at least some Bcl-2 proteins, including Bax and BNip3 (Tsunoda *et al.* 1999, Sundarajan & White 2001). Interestingly, induced mRNA expression of TNF- α (Feuerstein & Barone 1994, Dziewulska & Mossakowski 2003) and p53 (van Lookeren Campagne *et al.* 1998, Zu *et al.* 2002) has been reported early during focal cerebral ischaemia, and might explain the almost parallel induction of Bax and Nix responses observed following permanent and transient focal cerebral ischaemia in this thesis.

In vitro (see Chapters 3, -4 and -5) and *in vivo* studies (see Chapters 6 and -7), demonstrated that changes in subcellular distribution of a given protein did not necessarily correlate with total protein expression. For example, in some studies ischaemia was associated with reduced cytosolic levels of a protein, but total protein expression remained unaltered and a concomitant mitochondrial accumulation of this protein was not observed. This might be explained by re-distribution of Bax, Bad, Nix or the Nix-like protein to intracellular membranes, other than the mitochondria. Support for this proposal comes from several studies where Bax (Yamaguchi *et al.* 2003), BNip3 and Nix (Boyd *et al.* 1994) have been reported to localise to the endoplasmic reticulum (ER) and the nucleus. Furthermore, Bax, Nix and BNip3 were reported to retain, at least some, apoptotic activity when targeted to non-mitochondrial sites (Mandal 1998, Scorrano *et al.* 2003). Bcl-2 and Bcl-X_L, have also been reported to associate with the nuclear envelope and the ER (Givol *et al.* 1994, Krajewski *et al.* 1993, Lithgow *et al.* 1994) and more importantly, appear capable of inhibiting apoptotic cell death at these organelles (Lee *et al.* 1999, Zhu *et al.* 1996). Therefore, although this thesis only investigated the activity of this protein family at the mitochondria

literature suggests that these molecules likely transduce the apoptotic signal via other sites, in addition to the mitochondria.

More recently, Ku70, a 70 kDa subunit of the Ku complex, which has an important role in DNA double-strand break repair in the nucleus, was identified as a novel regulatory mechanism in Bax signalling (Sawada *et al.* 2003 a, b). Sawada *et al.* (2003a) reported that Ku70 interacts with Bax in the cytosol through the carboxyl terminal of Ku70 and the amino terminus of Bax, thus preventing Bax translocation to the mitochondria and subsequent activation of the intrinsic cell death cascade. Peptides generated from the Bax-binding domain of Ku70, were further observed to inhibit Bax-mediated apoptosis induced by staurosporine, UVC irradiation and several anti-cancer drugs in several cell types (Sawada *et al.* 2003b). These findings support staurosporine-treated human SH-SY5Y neuroblastoma cells investigated in the present study to be a suitable model to further characterise Bax regulatory mechanisms. Since the best characterised mechanism of action for the Bcl-2 family at present is control over mitochondrial integrity, and our understanding this regulatory mechanism is somewhat confusing to say the least, it might be anticipated that elucidation of the entire Bcl-2 family control over cell fate will require some time.

WHICH CELL TYPE EXPRESSED BAX, BAD, NIX & CLEAVED CASPASE-3?

Since thionin staining of brain sections after 24 h permanent ischaemia failed to detect more than a few surviving neurones, it might seem unlikely that the maximal pro-apoptotic Bcl-2 family responses, cleaved caspase-3 immunoreactivity and apoptotic bodies, all indicative of apoptotic cell death were expressed within neurones. Also, the finding that peak pro-apoptotic responses correlated with maximal ischaemic cell death in the core striatal lesion, further questions whether neurones might be dying by apoptosis in these experimental models of focal cerebral ischaemia. In contrast, subsequent staining of the same brain sections with H&E revealed many more surviving cells with a neuronal-like morphology in the ischaemic hemisphere. Further suggesting that neurones might retain residual energy at such time points to enable activation of the ATP consuming intrinsic apoptotic cell death pathway, a recent study in mice demonstrated that Akt co-localised with NeuN, a neuronal marker, in the ipsilateral hemisphere, 5 h after onset of transient (1 h) MCA occlusion focal ischaemia; at a time where a morphologically mature lesion was reported (Noshita *et al.* 2001a).

Another key question remains; was the observed increase in cleaved caspase-3 immunoreactivity a result of the increased pro-apoptotic Bcl-2 family protein response, which would require activation of the

apoptosome and caspase-9 cleavage – an ATP consuming event, or was it the direct result of death receptor activation (possibly through TNF α signalling) and caspase-8-mediated caspase-3 cleavage. Or even, a result of activation of both pathways. Evidence to support the activation of both pathways during permanent and transient ischaemia has been provided (Benchoua *et al.* 2001, Mouw *et al.* 2002). Alternatively, it might be predicted that early during ischaemia, where ATP levels are relatively well preserved, the observed pro-apoptotic Bcl-2 family response might have been initiated. However, as energy levels were depleted, particularly following 24 h permanent ischaemia, it might be proposed that the cells aborted the energy-consuming ‘intrinsic’ apoptotic cell death pathway and entered the ‘extrinsic’ pathway through activation of the BH3-only protein, Bid (Lutter *et al.* 2001, Roucou *et al.* 2002). Bid activity was not investigated in this thesis due to high non-specific binding of available antibodies. However, increased pro-apoptotic Bid activity has been reported in several studies of focal cerebral ischaemia (Yin *et al.* 2002, Ferrer *et al.* 2003) and might explain why increased cleaved caspase-3 expression was detected in the core ischaemic lesion; an area conventionally considered to have little to no ATP and to have cells that have died primarily by necrosis (Siesjö *et al.* 1992). Alternatively, since the increased pro-apoptotic response, not only correlated with increased ischaemic damage, but also with an increased inflammatory response, it might be suggested that the observed apoptotic changes might have been expressed by either the monocytes, macrophages or neutrophils all identified within the core lesion. Accumulative evidence suggests leukocytes may play a key role in secondary brain damage that is reported during reperfusion (Jean *et al.* 1998). Initial studies used a myeloperoxidase assay and reported maximum levels of leukocyte infiltration 48-72 hours following permanent MCA occlusion, accumulating within the core lesion 7 days later (Garcia *et al.* 1974). However, more recently, leukocyte infiltration was observed after 1-4 h reperfusion, reaching maximal levels within 24-72 h (Garcia 1994, Hallenbeck 1996). This is in agreement with stroke patients who demonstrate neutrophil infiltration within the first 18 hours after an acute ischaemic stroke (Bednar *et al.* 1991). Supporting this proposal, Bax expression and other markers of apoptosis have been reported in neutrophils (Zhao *et al.* 2001, Loeffler *et al.* 2003) and other non-neuronal cell types such as oligodendrocytes (Mabuchi *et al.* 2000, Osterhout *et al.* 2002). Finally, the observed pro-apoptotic Bax, Bax, Nix, Nix-like protein responses as well as the Akt survival signalling response might have been expressed in different cell types, or sub-populations of neurones with varying resistance to ischaemia.

TIME TO RE-DEFINE THE TERMS 'APOPTOSIS' & 'NECROSIS' ?

The term 'apoptosis' describes an active process of cellular destruction originally contrasted morphologically with necrosis. The mistaken equivalence of the terms apoptosis and 'programmed cell death' has caused confusion and implicated that apoptosis is an identifiable therapeutic target rather than a name of a type of cell death. The belief that the cell death process called 'apoptosis' is triggered in a variety of neurological disorders made neuronal apoptosis a subject of intense research and a prime target for therapeutic intervention (Bredesen *et al.* 1995, Kinloch *et al.* 1999, Nicotera *et al.* 1999b, Wang *et al.* 2000). A recent review by Sowter (2002) raised the questions as to exactly what is apoptosis. It was suggested that what has become considered a specific and ubiquitous mechanism by which cells destroy themselves might be better considered a poorly defined name applied to a variety of cell death processes. The idea of apoptosis was originally conceived because cells dying in a variety of conditions exhibited morphological features suggestive of an active process of cellular destruction (Kerr 1972). Moreover, the presence of nuclear pathology is often interpreted as evidence that apoptosis has taken place, even when the nuclear morphology is not comparable to physiological apoptosis (Clark *et al.* 1999, Colbourne *et al.* 1999, Fujikawa *et al.* 2000). Some investigators define necrosis as a process of immediate and catastrophic cell death in which no cellular processes are involved (Hetts *et al.* 1998), whereas others define the term as an active cell death process with an initially cytoplasmic pathology (Sloviter *et al.* 1996, Clark *et al.* 1999, Colbourne *et al.* 1999, Fujikawa *et al.* 2000). The term 'necrosis' is now generally equated with infarction, loss of neurones, glia and endothelial cells following focal cerebral ischaemia, but does not aid characterisation of the mechanism of cell death. In fact, there is no definition for necrosis or apoptosis that precisely describes any particular type of readily definable cell death. Apoptosis is not synonymous with programmed cell death (Lockshin & Williams 1965, Ellis *et al.* 1986) because apoptosis was not originally described mechanistically, but conceptually and morphologically (Kerr *et al.* 1972). Although the biochemical processes that presumably cause the morphological features of dying cells have been inferred to be potential apoptosis-related 'programs' (Arends *et al.* 1990), many biochemical changes occur in actively dying cells, none of which are known to be unique to the developmental and physiological type of cell death originally called apoptosis (Corcoran *et al.* 1994). Although the morphology of ischaemic cell death does not resemble the morphology of classical apoptosis (Kerr *et al.* 1972), the observation that ischaemic cell death involves activation of cellular processes after reperfusion and before cell death has led many investigators to conclude that, at least some forms of, ischaemic cell death should be called apoptosis (Choi *et al.* 1996, Du *et al.* 1996, MacManus *et al.* 1997).

Sloviter (2002) recently suggested that instantaneous cell death in which the cell plays no role in its own demise should be referred to as 'passive cell death' rather than necrosis. Passive cell death would refer to

an immediate mechanism which would not involve cellular activity. In this scenario, passive cell death would be of no pharmacological relevance in stroke research because the immediate cell death would offer no therapeutic window. In contrast, Sloviter suggested that the term 'active cell death' (ACD) would replace the names apoptosis and programmed cell death. ACD would include any lethal process requiring any active intracellular processes for death to result. By definition, ACD would require time to occur and would be a potential therapeutic target. 'Immediate ACD' would describe any process that begins within minutes after the triggering event, whereas 'delayed ACD' would exhibit a delayed initiation phase. Based on these suggested definitions, it might be suggested that the observed pro-apoptotic Bcl-2 family response with concomitant increased caspase-3 cleavage might reflect a delayed ACD response, which might be a potential therapeutic target in stroke.

CONCLUSIONS

This thesis was designed to investigate the role of the pro-apoptotic Bcl-2 family proteins in focal cerebral ischaemia and has tackled many issues, such as (i) the controversial mechanism(s) of action of Bax and other pro-apoptotic Bcl-2 family proteins, (ii) identified several *in vitro* and *in vivo* novel regulatory mechanisms for Akt and Nix, (iii) confirmed the mitochondria to be a potentially critical site of action for the Bcl-2 family and (iv) provided evidence supporting a role for pro-apoptotic Bcl-2 family of proteins in stroke. However, as is often the case, these findings raised even more questions regarding the mechanism of action of this protein family. Based on evidence provided in this thesis, and supporting literature, a 'revised' schematic diagram outlining the pathophysiology of focal cerebral ischaemia has been constructed (*Figure 8.3; compare with Figure 1.5.2*).

These studies demonstrated that late reperfusion, when initiated after 2 h MCA occlusion; the minimal time to produce irreversible damage might be detrimental and propagate necrotic-like cell damage. This might explain why apoptosis was observed following 24 h transient (1 h MCA occlusion) and permanent ischaemia, but not after 24 h transient ischaemia following an initial 2 h MCA occlusion period. However, the time course of lesion progression and underlying cell death mechanisms differed between permanent and transient focal cerebral ischaemia in the rat. In addition reperfusion, when initiated within 1 h MCA occlusion, appeared to delay the progression of histological and biochemical features of ischaemic damage compared to 6 h and 24 h permanent ischaemia. A progressive pro-apoptotic Bax, Nix and Nix-like protein response was observed with increased ischaemic duration and reperfusion duration. Interestingly, a novel intrinsic Akt-mediated survival mechanism, which involved inhibition of the pro-apoptotic Bad

response, was detected during early ischaemia and with prolonged reperfusion duration. These studies provide convincing evidence to support a role for the pro-apoptotic Bcl-2 family proteins Bax, Bad, Nix and the novel Nix-like protein, in the pathophysiology of focal cerebral ischaemia and have identified this protein family is a justifiable target in stroke research.

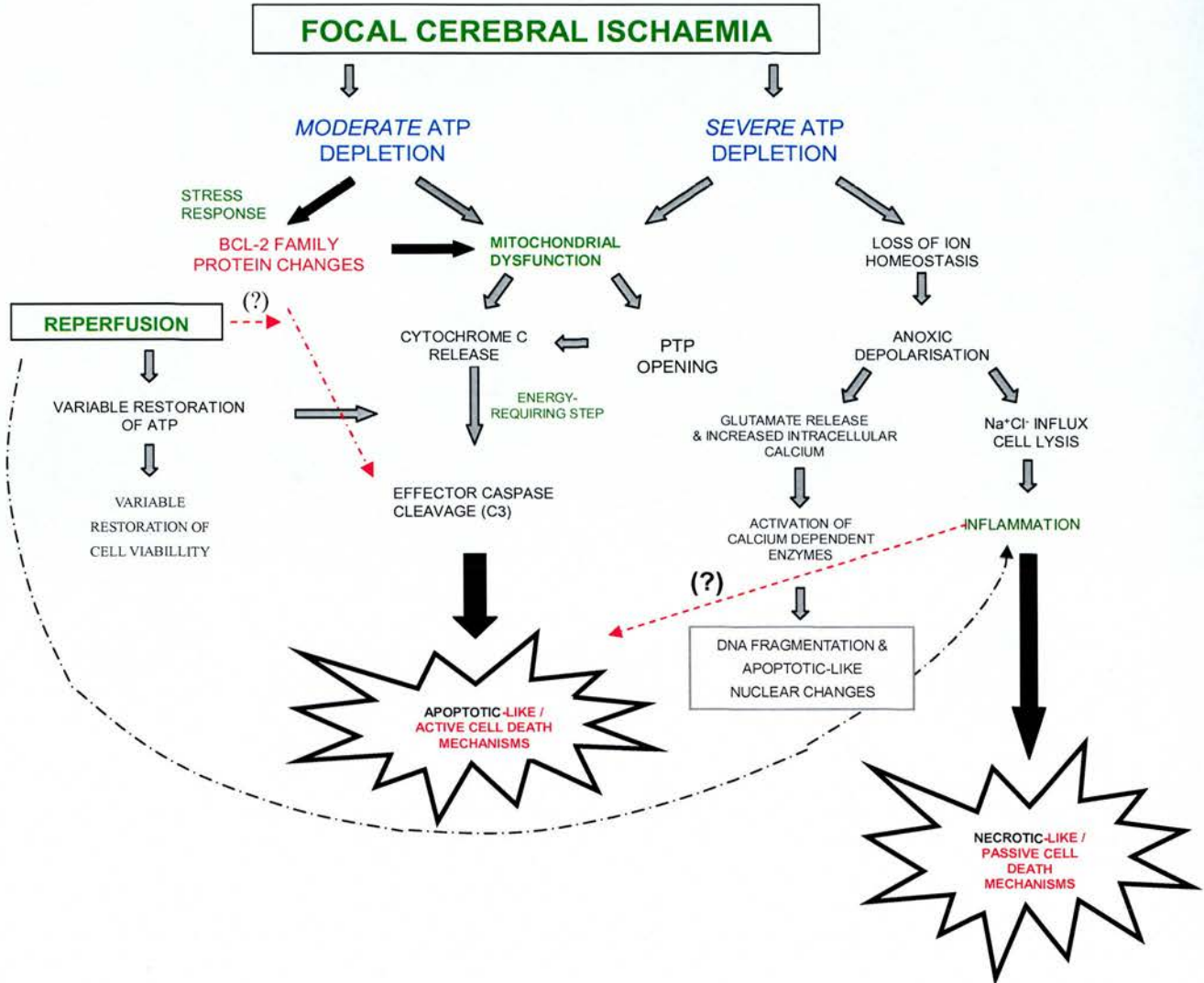


Figure 8.3: Schematic diagram of the pathophysiology of focal cerebral ischaemia in the rat.

APPENDICES

APPENDIX A: HISTOLOGY

Thionin staining methods: Sections (20µm) were placed in Histoclear initially to remove lipid and rehydrated through an alcohol series before being placed in thionin (0.03 %, Sigma) for 20 min. Sections were differentiated in 0.2 % acetic acid, dehydrated through the alcohol series and mounted in Xylene with DPX (distrene, plasticiser and xylene, BDH Chemicals Ltd, UK).

Haematoxylin & Eosin staining methods: Thionin-stained sections were de-stained by first removing the coverslips in Xylene. Thionin staining was subsequently removed by taking brain sections to water through an alcohol series (100% 2x2 min, 90%, 80% (2 min each) 70% - until all thionin staining was removed. Sections were washed in water, placed in Harris' Haematoxylin (Merck Biosciences) for 5 min and differentiated in acid alcohol (1% HCl in 70% alcohol). After washing in water, sections were placed in Eosin (0.5%) for 5 seconds and placed in bluing agent, Scott's Tap water substitute (3.5g Na₂HCO₃, 20g Mg₂SO₄ in 1L H₂O) for 20 min to darken the staining of the nucleus. Sections were re-hydrated and mounted as with thionin stained sections.

Calculation of damage

The trapezoidal rule was chosen to calculate the volume as it accounted for the unequal distances between the template levels chosen (Rosen & Harry, 1990). Sections were examined under a light microscope (Leitz) and the ischaemic damage annotated onto templates representing 9 pre-determined stereotaxic levels. Templates (*Appendex B*) were simplified from The Rat Brain in Stereotactic Coordinates (Paxinos & Watson 1986). This method of calculating the volume of damage accounts for distortion or shrinkage that may occur following perfusion fixation and oedema associated with the lesion. The damage was quantified using a microcomputer imaging device (MCID, Imaging Research Inc.) and volume calculated according to the trapezoid model. This model has been reported to give consistently accurate results when calculating the volume of objects with unequal distances between sampled sections (Rosen & Harry 1990).

APPENDIX B: COMPUTERISED DIGITAL MORPHOMETRY

Templates were modified from 'The Rat Brain in Stereotaxic Co-ordinates', (Paxinos & Watson, 1986). The 9 templates were chosen to cover the area of a characteristic MCA occlusion damage from +4.2 to -7.8 from Bregma (+4.2, +2.2, +1.2, +0.2, -0.8, -1.8, -3.8, -5.8, -7.8) with easily recognisable anatomical landmarks. The levels were roughly based on those already in use by other investigators who demonstrated accurate assessment of the volume of damage with 8 levels without a loss of accuracy (Osborne et al., 1987). This method of calculating the volume of damage also accounts for distortion or shrinkage that may occur following perfusion fixation and oedema associated with the lesion.

Stained sections were examined by light microscopy and the damage annotated onto the templates in pencil initially and then coloured dark blue. The templates were digitised using the MCID M5+ image analyser (version 4.00 Rev 1.5) and the total area of damage computed for 82 regions of the rat brain (37.7 pixels.mm⁻¹ on the horizontal and 36.6 pixels.mm⁻¹ on the vertical). Values obtained were transposed into an Excel workbook and the volume of damage calculated using the trapezoid rule. This model has been reported to give consistently accurate results when calculating the volume of objects with unequal distances between sampled sections (Rosen & Harry 1990). Damage calculated was grouped into cortex, striatum and mid-line (all non-cortical and non-striatal regions) regions. Data was graphed using SigmaPlot 8.0 and analysed statistically using SigmaStat 4.

TRAPEZOID RULE
$$V = \Sigma (x_{i+1} - x_i) [(y_i + y_{i+1})/2]$$

Where V is volume, x_i is the distance orthogonal to the plane of the i -th section and y_i is the cross sectional area of the i -th section (Rosen & Harry 1990).

Page IV: Description of the 82 regions of the 9 rat stereotaxic templates used for computerised digital morphometry analysis.

Page V: Sample of the stereotaxic templates used to score ischaemic damage.

Page VI: Rat stereotaxic templates showing division of the regions into cortex, striatum and mid-line.

Page VII: Sample of the Excel worksheet used to calculate volume of damage from the raw data.

Levels 1-8 represent the volume of damage calculated using the Trapezoid rule between two stereotaxic levels. For example, level 1 represents damage between Bregma levels +4.2 and +2.2.

RAT BRAIN REGIONS

BREGMA +4.2

Medial, ventral & ventrolateral orbital cortex;
cingulate cortex areas 1 & 3
Frontal cortex area 2
Agranular insular cortex
Lateral orbital cortex
Anterior olfactory nuclei

BREGMA +2.2

Frontal cortex area 2
Frontal cortex area 1 & 3
Parietal cortex area 1
Agranular insular cortex; ventrolateral & lateral
orbital cortex; claustrum
Piriform cortex
Basal forebrain
Accumbens nucleus
Caudate putamen
Cingulate cortex areas 1 & 3, infralimbic cortex,
dorsal peduncular cortex, tenia tecta

BREGMA +1.2

Cingulate cortex areas 1 & 2
Frontal cortex area 2
Frontal cortex area 1; forelimb area of cortex
Parietal cortex area 1
Agranular, dysgranular & granular insular cortex;
claustrum
Piriform cortex
Septum; basal forebrain
Accumbens nucleus
Caudate putamen

BREGMA +0.2

Cingulate cortex areas 1 & 2
Frontal cortex area 2
Frontal cortex area 1; forelimb area of cortex
Parietal cortex area 1 & 2
Agranular, dysgranular & granular insular cortex;
claustrum
Piriform cortex; dorsal endopiriform cortex
Lateral septum; basal forebrain
Caudate putamen
Medial septum

BREGMA -0.8

Cingulate cortex areas 1 & 2
Frontal cortex area 2
Frontal cortex area 1; forelimb & hindlimb area of
cortex
Parietal cortex area 1 & 2

Agranular & granular insular cortex; claustrum
Piriform cortex
Anterior hypothalamic area
Globus pallidus
Caudate putamen

BREGMA -1.8

Retrosplenial cortex
Frontal cortex area 2
Frontal cortex area 1; hindlimb area of cortex
Parietal cortex area 1 & 2
Perirhinal cortex
Piriform cortex
Hypothalamic nuclei & amygdaloid nuclei
Thalamic nuclei
Globus pallidus
Caudate putamen

BREGMA -3.8

Retrosplenial cortex
Occipital cortex
Parietal cortex area 1 & 2
Temporal cortex
Perirhinal cortex
Piriform cortex
Amygdaloid nuclei
Caudate putamen
Cerebral peduncle
Hypothalamic nuclei
Thalamic nuclei
Hippocampus
Habenula nucleus

BREGMA -5.8

Retrosplenial cortex
Occipital cortex
Temporal cortex
Perirhinal cortex
Entorhinal cortex
Hippocampus & amygdaloid nuclei
Deep mesencephalic nuclei
Substantia nigra
Central gray

BREGMA -7.8

Retrosplenial cortex
Occipital cortex
Temporal cortex
Perirhinal cortex
Entorhinal cortex
Tegmental nuclei
Central gray
Raphe nuclei
Paralemniscal nuclei

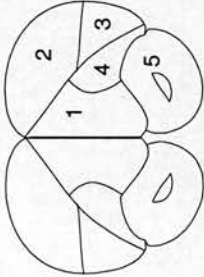
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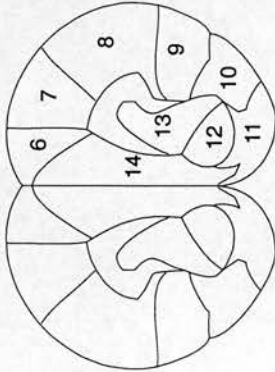
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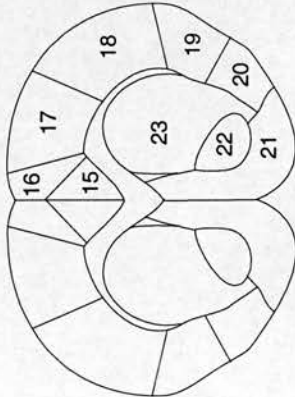
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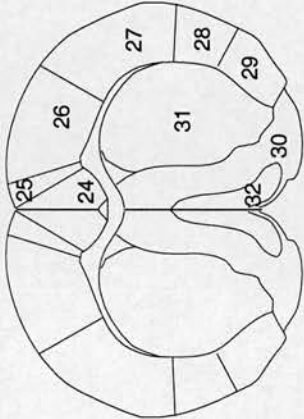
+2.2



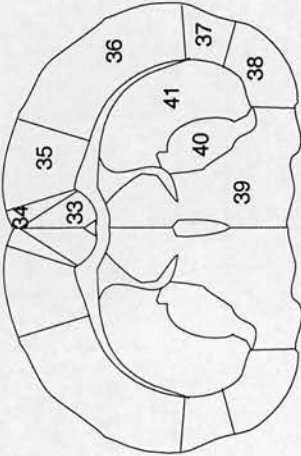
+1.2



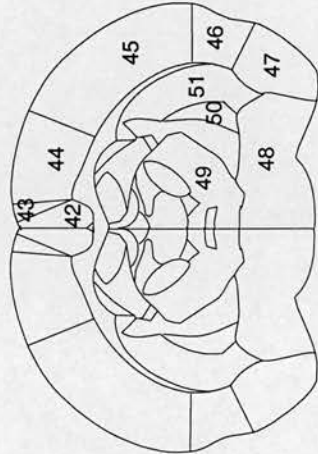
+0.2



-0.8

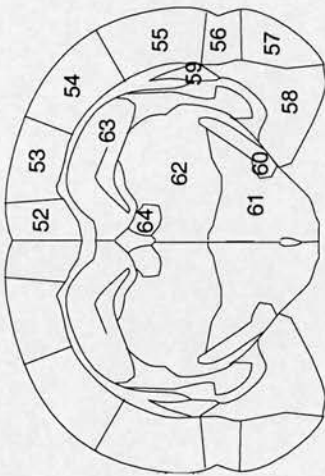


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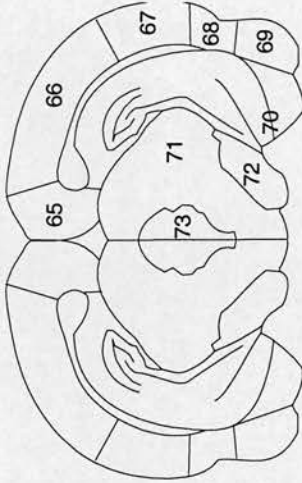


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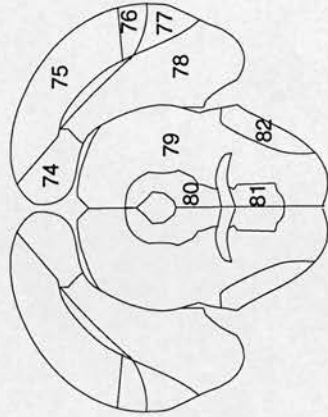
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-5.8

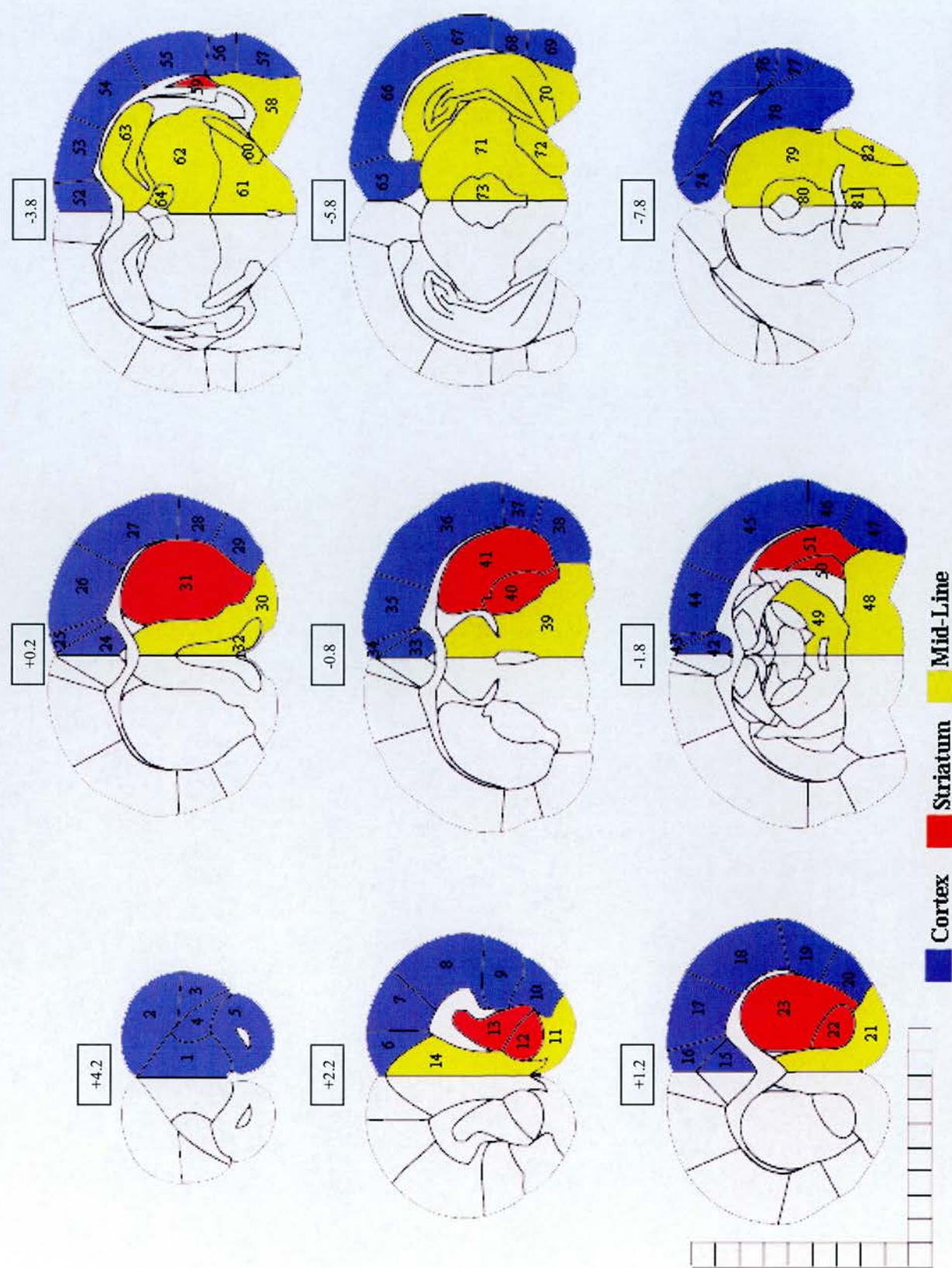


-7.8



RAT STEREOTACTIC LEVELS: Bregma +4.2; +2.2; +1.2; +0.2; -0.8; -1.8; -3.8; -5.8; -7.8

Signed: _____ Date: _____



Optimization of the Transient (2 h MCA occlusion) Ischaemic Stroke Model in Rats

REGION	LEVEL	Animal 1	Animal 2	Animal 3	Animal 4	Animal 5	Animal 6	Animal 7
CORTEX (mm ³) (Ipsilateral Values Only)	1	17.57	0.00	16.71	15.82	20.06	19.63	16.61
	2	14.58	0.01	14.64	14.69	16.53	14.91	16.02
	3	16.17	0.02	16.24	16.64	17.46	15.34	16.55
	4	17.91	0.03	17.57	17.92	18.35	17.00	17.38
	5	19.04	0.05	19.38	18.24	19.46	18.73	19.62
	6	33.26	0.07	34.32	32.83	35.30	29.91	30.82
	7	25.65	0.00	25.69	21.09	24.96	20.43	20.12
	8	13.88	0.00	14.27	5.71	11.74	13.29	13.12
TOTAL		158.06	0.17	158.81	142.94	163.86	149.23	150.22
STRIATUM (mm ³) (Ipsilateral Values Only)	1	0.61	0.86	0.94	0.77	1.49	1.21	1.86
	2	4.47	3.45	4.51	4.64	5.47	4.01	4.65
	3	9.58	7.18	10.32	10.26	11.31	8.91	8.51
	4	10.44	7.14	12.34	10.67	11.84	10.92	10.83
	5	7.15	3.96	8.47	7.04	7.68	7.83	8.45
	6	4.55	2.20	5.23	5.12	5.23	5.23	5.21
	7	0.29	0.25	0.40	0.35	0.40	0.40	0.40
	8	0.00	0.00	0.00	0.00	0.00	0.00	0.00
TOTAL		37.09	25.04	42.20	38.85	43.41	38.49	39.92
MID-LINE (mm ³) (Non Str/Cort Regions- Ipsilateral Values Only)	1	0.00	0.00	0.00	0.00	0.00	0.00	0.00
	2	0.07	0.00	0.05	0.01	0.85	0.00	0.72
	3	0.24	0.00	0.52	0.02	1.41	0.00	1.12
	4	1.52	0.00	1.22	0.01	1.07	0.22	2.96
	5	3.05	0.00	1.54	0.83	2.17	0.90	3.66
	6	8.67	0.00	2.49	7.14	8.78	1.38	5.93
	7	7.10	0.00	0.98	5.48	5.47	0.04	3.80
	8	1.82	0.00	0.07	0.00	0.00	0.01	0.08
TOTAL		22.47	0.00	6.87	13.49	19.75	2.54	18.26
OVERALL TOTAL		217.62	25.21	207.89	195.28	227.03	190.25	208.41

MEAN	SD	SEM
15.20	6.89	2.60
13.05	5.80	2.19
14.06	6.22	2.35
15.16	6.69	2.53
16.36	7.21	2.72
28.07	12.49	4.72
19.70	9.03	3.41
10.29	5.40	2.04
131.90	58.51	22.11

1.10	0.44	0.17
4.45	0.62	0.23
9.44	1.37	0.52
10.60	1.67	0.63
7.23	1.54	0.58
4.68	1.12	0.42
0.36	0.06	0.02
0.00	0.00	0.00
37.86	6.06	2.29

0.00	0.00	0.00
0.24	0.37	0.14
0.47	0.58	0.22
1.00	1.06	0.40
1.73	1.30	0.49
4.91	3.59	1.36
3.27	2.92	1.10
0.28	0.68	0.26
11.91	8.86	3.35
181.67	70.11	26.50

APPENDIX C: RUNNING GELS & WESTERN BLOTTING

RUNNING SDS-PAGE GELS

Place gel in gel tank and secure

If only running one gel, secure an aluminium back plate to the other side

Fill the middle gel chambers with running buffer, add running buffer to the bottom buffer chamber

Wash wells with running buffer

Prior to loading heat samples to 95°C for 5 min then pulse spin

Load samples and check buffer level in top/middle buffer chamber

Run at 70V constant through stacking gel (~ 30 min)

Increase voltage to 150V through separating gel

Stop gel running when dye front reaches the bottom of the gel (~ 2 h)

Transfer gel to gel box containing transfer buffer and soak gel for ~10 min

Hybond C – pre-wet in MQ H₂O then into transfer buffer

Soak membrane in transfer buffer for ~ 10 min

Sandwich membrane and gel between two wet filter papers so proteins (-) in the gel are repelled from negative electrode; 15V for ~1 h

Block membrane for 1 h at room temperature – 5% blotto in TBS 0.1% Tween pH 8

Incubate with 1st antibody diluted in block – overnight at 4 °C

Wash 1 x 10 min; 2 x 5 min in TBS 0.1% Tween pH 7.4

Incubate with 2nd antibody diluted in block 1-2 hrs at room temperature

Wash 1x 10 min, 2x 5min in TBS 0.1% Tween pH 7.4, Develop using ECL (Amersham)

BUFFERS FOR RUNNING SDS GELS & WESTERN BLOTTING

Running Buffer

1x Tris/Glycine buffer, 0.1% SDS

10x Tris/Glycine buffer 100ml

10% SDS 10ml

MQ H₂O to 1L

10x Tris/Glycine Buffer

250mM Tris, 1.92M glycine

Trizma base 30g

Glycine 142.6g

MQ H₂O to 1L

Stripping Buffer

0.2M glycine, 1% Tween-20,

0.1% SDS pH 2.2

1M glycine pH 2.2 2ml Tween-20

100μl 10% SDS 100μl

MQ H₂O to 10ml

Transfer Buffer

1x Tris/Glycine buffer, 20% methanol

10x Tris/Glycine buffer 100ml

methanol 200ml

MQ H₂O to 1L

10x Tris/Glycine Buffer

250mM Tris, 1.92M glycine

Trizma base 30g

Glycine 142.6g

MQ H₂O to 1L

10x TBS

200mM Tris, 730mM NaCl, pH 8

Trizma base 24.2g

NaCl 80g

Adjust pH to 8

MQ H₂O up to 1L

Block solution

5% blotto, TBS, 0.1% Tween-20, pH 8

blotto 5g

TBS 0.1% Tween-20 pH 8 to 100ml

Adjust to pH 8

(All reagents from Sigma)

SDS ACRYLAMIDE GELS

12 % Acrylamide Gel

8 ml 30 % Acrylamide/Bis solution 29:1 (3.3 % C) (BioRad)

4 ml 1.5 M Tris pH 8.8

0.16 ml 10 % SDS

16 µl TEMED (Sigma)

120 µl 10 % ammonium persulphate

3.7 ml MQ H₂O

4 % Acrylamide Stacking Gel

1.3ml 30 % Acrylamide/Bis solution 29:1 (3.3 % C) (BioRad)

1.25ml 1 M Tris pH 6.8

10% SDS

10µl TEMED

75µl 10 % ammonium persulphate

7.3ml MQ H₂O

For both 12 % and 4 % gels stock solutions of acrylamide, Tris and SDS were made and stored at 4°C. All stock reagents from Sigma, unless otherwise stated. To pour the gels the appropriate volume of TEMED and freshly made ammonium persulphate was added. Gels were poured between Hoefer glass plates (Amersham Pharmacia) with a 10 or 15 well comb.

Sample Buffer

2 ml Glycerol

2 ml 10 % SDS

0.25 mg Bromophenol Blue

2.5 ml stacking gel buffer (6.06 g Tris in 100 ml, 4 ml 10 % SDS pH 6.8)

0.5 ml β-mercaptoethanol

APPENDIX D: MTS CELL VIABILITY ASSAY

Cell viability was assessed using the Cell Titer 96® Aqueous One Solution Assay (Promega). The assay is based on the MTS tetrazolium compound [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulphophenyl)-2H-tetrazolium] being bio-reduced by active cells into a coloured formazan product. This is accomplished by NADPH or NADH which is produced by dehydrogenase enzymes in metabolically active cells.

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